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Wild Chinese giant salamander persist in Zhangjiajie, Hunan, China: Evidence from 10 years of monitoring

Zhiqiang Liang [a,e], Qianqian Wu [b,*], Li Zou [a], Zhiyong Deng [c], Jinxin Zhou [d], Xuan Xie [a], Linmei Han [a], Chongrui Wang [a], Xunhua Liu [a], Mingqiu Liu [a], Qiwei Wei [e]

[a] Hunan Fisheries Research Institute and Aquatic Products Seed Stock Station, Hunan Academy of Agricultural Sciences, Changsha 410153, China
[b] Graduate School of Human Development and Environment, Kobe University, 3-11 Tsurukabuto, Nada-ku, Kobe City, Hyogo 657-8501, Japan
[c] Administrative Office of The National Giant Salamander Nature Reserve of Zhangjiajie, Hunan 410004, China
[d] Institute of Industrial Science, The University of Tokyo, Kashiwanoha 5-1-5, Kashiwa, Chiba 277-8574, Japan
[e] Key Laboratory of Freshwater Biodiversity Conservation, Ministry of Agriculture of China, Yangtze River Fisheries Research Institute, Chinese Academy of Fishery Sciences, Wuhan 430223, China

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ABSTRACT

As a critically endangered species, the Chinese giant salamander (CGS) is of both theoretical and practical importance in conservation biology. This study applied a multi-scale, integrated approach to characterize its reproductive ecology, combining long-term field monitoring and molecular methods. From 2005–2014, fixed-site surveys were conducted at nine representative breeding caves in Zhangjiajie, China. In 2023, environmental DNA (eDNA) technology was introduced to reassess the current distribution of wild populations. Results showed marked differences in larval emergence among caves, with annual averages ranging from 15 to 822 individuals. Emergence patterns were primarily influenced by shading, river width, flow rate, and dissolved oxygen. The timing of emergence displayed spatiotemporal gradients from December to February, as well as a distinct diel rhythm, with significantly higher emergence between 19:00–23:00. Behavioral observations recorded the color-changing process of CGS larvae upon exiting the cave. The body color darkened rapidly under stronger illumination, and typically reached completion within 4–12 h. Notably, eDNA analyses confirmed the persistence of species-specific DNA fragments in several historical sites where no larvae had been observed for over five years. These findings suggest the continued survival potential of wild individuals in areas previously considered unoccupied. By integrating conventional ecological monitoring with eDNA analysis, this study addresses critical gaps in knowledge of the reproductive ecology and spatial persistence of the CGS. The combined framework establishes a robust foundation for targeted conservation strategies and future management of this iconic amphibian.

1. Introduction

The Chinese giant salamander (*Andrias davidianus*; CGS), commonly known as the "WawaYu" (baby fish), is the largest extant amphibian on Earth, conferring significant ecological, cultural, and historical research value (Gao and Shubin, 2003; Zhu et al., 2014).

* Correspondence to: Graduate School of Human Development and Environment, Kobe University, 3-11, Tsurukabuto, Nada-ku, Kobe city, Hyogo 657-8501, Japan.
E-mail addresses: wuqq@people.kobe-u.ac.jp, wuqian1124@yahoo.co.jp (Q. Wu).

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Historically, this species exhibited a broad distribution encompassing the Yangtze, Yellow, and Pearl River basins and their tributaries (Song, 1986; Liu, 1989; Yang, 1991; Ye, Li and Hu, 1993; Wang et al., 2004), occurring at elevations exceeding 1500 m (Wang et al., 2004). Currently classified as Critically Endangered by the International Union for Conservation of Nature (IUCN SSC Amphibian Specialist Group, 2023), it is also designated as a National Second-Class Protected Species in China (National Forestry and Grassland Administration of China, 2025). Recent taxonomic advances have formally recognized multiple species, including the CGS (*A. davidianus*) (Blanchard, 1871), Huanan giant salamander (*A. sligoi*) (Turvey et al., 2019), Jiangxi giant salamander (*A. jiangxiensis*) (Chai et al., 2022), and Qimen giant salamander (*A. cheni*) (Gong et al., 2023). However, with the exception of *A. jiangxiensis*, the precise distribution ranges and population sizes of other *Andrias* species remain uncertain, posing significant conservation challenges (Chai et al., 2022). Wild CGS individuals, subjected to overfishing, habitat destruction, and environmental pollution, have declined dramatically, experienced severe range contraction, and face a precarious survival status (Dai et al., 2009; Zhang et al., 2002). Notably, their strong preference for inhabiting remote karst cave systems significantly complicates consistent field surveys (Barata et al., 2017; Su, Yu and Ma, 2009). Consequently, ecological studies on wild individuals within natural breeding caves—particularly concerning natural reproduction and habitat utilization—remain scarce. Critically, cave breeding behavior in wild individuals remains unconfirmed, and the color change patterns of larvae after emerging from breeding caves lack systematic documentation.

Situated in northwestern Hunan Province, Zhangjiajie is a significant native habitat of the CGS. It established China's first national-level nature reserve dedicated to this species—The National Giant Salamander Nature Reserve of Zhangjiajie. Renowned for its karst topography, this region features extensive limestone cave networks and subterranean river systems (Luo, Liu and Zhang, 2009). These rivers, formed by converging groundwater and interconnected with the cave networks, provide clear, oxygen-rich waters that constitute an ideal habitat and abundant food resources for the CGS (Luo, Liu and Zhang, 2009). Historically, Zhangjiajie supported extremely abundant wild populations of CGS and represents one of the core native habitats of the species (Luo, Liu and Zhang, 2009). Consequently, selecting Zhangjiajie as a study site not only reflects the current status of wild CGS individuals in China but also provides a highly representative system for ecological research. However, due to the species' nocturnal behavior and specialized cave-dwelling ecological traits, research on its natural breeding ecology in caves remains limited. However, research on their natural breeding caves remains scarce due to the species' nocturnal behavior and specialized cave-dwelling ecology. This gap hinders understanding of their life history, particularly the "larvae emergence" process during early development, and impedes the development of efficient, accurate scientific methods for assessing current wild individual status.

To systematically investigate the characteristics of wild CGS in their early development phase in natural breeding caves, this study employed fixed-point observation to monitor the complete larval emergence process within their life cycle. Previous studies have shown that their reproductive behavior shows distinct seasonality, typically occurring intensively in summer and autumn (Luo et al., 2021; Jiang, Tian and Zhang, 2022), while the stable environment within caves (such as constant water temperature, low light, and high humidity) may provide ideal conditions for egg hatching and the early development of larvae (Luo et al., 2009a, b). In the early developmental stages, the CGS larvae, commonly called “Da Ni Miao” (baby CGS), typically passively disperse from the caves with the water flow after hatching, and this process is referred to as “larval emergence” (Liang, 2015). Previous studies have described that newly hatched CGS larvae exhibit lighter coloration upon cave emergence (Liang, 2015). This trait is considered an anti-predator strategy and a form of phenotypic plasticity, where body color darkens during ontogeny in response to environmental exposure, such as light and substrate changes. Similar to *Oreolalax rhodostigmatus* (Zhu et al., 2018) and certain cave fish (Gross, Borowsky and Tabin, 2009), CGS larvae likely undergo physiological darkening triggered by external stimuli to adapt to varying habitats. However, due to the lack of long-term dynamic observations in natural breeding caves, ecological data on wild newly hatched CGS larvae remains a significant research gap.

Environmental DNA (eDNA) technology is increasingly adopted to clarify the distribution of the giant salamanders (Fukumoto et al., 2015; Hidaka et al., 2024). The rise of eDNA technology has brought a great revolution in monitoring aquatic species, and eDNA technology refers to detecting the total DNA information left by all organisms in the surroundings, including skin cells, excrement, and decomposed tissues present in the water (Ficetola et al., 2008). This method offers a highly sensitive and convenient way to rapidly discover target species through several steps including water sampling, DNA extraction, and PCR amplification, etc. (Goldberg et al., 2016; Lodge et al., 2012), which has already been successfully applied to monitor the distribution ranges of Japanese giant salamanders and CGS (Fukumoto et al., 2015; Zhou et al., 2024). Therefore, applying eDNA surveillance to Zhangjiajie’s cave systems may yield reliable data on wild individuals’ distribution and survival status, informing targeted habitat conservation strategies.

This study aims to reveal the early developmental characteristics and current distribution of CGS larvae through an interdisciplinary approach, while testing the hypothesis that recent non-detection reflects critically low population numbers rather than complete extinction of wild individuals. There are mainly four components: 1) Long-term Fixed-point Monitoring: Systematically observe caves where CGS larvae have been consistently seen over the past decade, documenting the spatiotemporal dynamics of larval individuals; 2) Ecological Behavior Research: Record detailed data on the timing of larval emergence, changes in larval numbers, and physiological traits (such as body color transitions); 3) Environmental Feature Analysis: Measure key water quality parameters (dissolved oxygen (DO), pH, water temperature, etc.) and other environmental factors of larval emergence sites to understand the ecological features of breeding caves; 4) Current Distribution Mapping: Collect water samples at cave outlets and nearby river systems, and assess the current distribution of CGS in the cave systems of Hunan province using eDNA technology. The innovation of this study lies in that it concentrates on the larval emergence patterns and physiological traits of wild CGS in naturally breeding caves in Zhangjiajie city in a systematic way. At the same time, eDNA technology offers a totally new pathway for non-invasive and efficient monitoring methods to comprehensively assess the status of wild individuals.

2. Material and methods

2.1. Experiment 1: Monitoring Natural Breeding Caves

2.1.1. Outflow Time, Number of individuals, and Physiological Changes of CGS larvae

To investigate the outflow time, quantity, and physiological changes of CGS larvae in natural breeding caves, researchers in this study, with the assistance of local authorities, carried out rescue capture of larvae using nylon nets (based on mesh size) at the outlets of caves. As every winter (from December to the next January) is the period when wild CGS larvae in Zhangjiajie emerge from their natural breeding caves, this study was conducted during the winters from 2005 to 2014. For nine natural caves (YZ, QYQ, STB, HK, CDX, WMY, LZT, ZC, and JBX Caves) (Fig. 1) (Appendix S1) where CGS larvae have been flowing out for a long time, the times of larval emergence each winter were estimated by the following formula in accordance with the method of Luo et al. (2009b). Statistical analysis, assuming independence based on the results, adopted the Kruskal-Wallis test for overall cave comparisons, followed by pairwise Wilcoxon rank sum tests with Holm-Bonferroni correction to adjust for multiple testing.

Resource quantity of CGS in the cave = Annual outflow number of larvae × 0.2976 (tail count) (Eq. 1) (Luo et al., 2009 b)

In addition, detailed observations were made at the outflows of YZ Cave (2012–2013) and WMY Cave (2011–2013), recording the time when CGS larvae emerged. Furthermore, the physiological changes (the process of body color change) were carefully documented at 10 min, 20 min, 50 min, 5 h, 12 h, and 15 h after exposure to external light. In early December 2012 (19:00–23:00), the light intensity at the YZ Cave outlet was measured using a digital illuminometer (LX1010, Sanpometer, China), with recorded values ranging from 10 to 200 Lux. Based on these field observations, three experimental light treatments (10–20, 50–80, and 100–150 Lux) were established in separate rooms using incandescent lamps. During the experiments, the illuminometer probe was positioned at a fixed distance of 5 cm from the larvae's dorsal skin. The time required for the body and tail to undergo darkening was recorded for each light intensity. Differences in darkening duration across treatments were analyzed using one-way ANOVA, followed by Tukey's HSD test for post-hoc multiple comparisons.

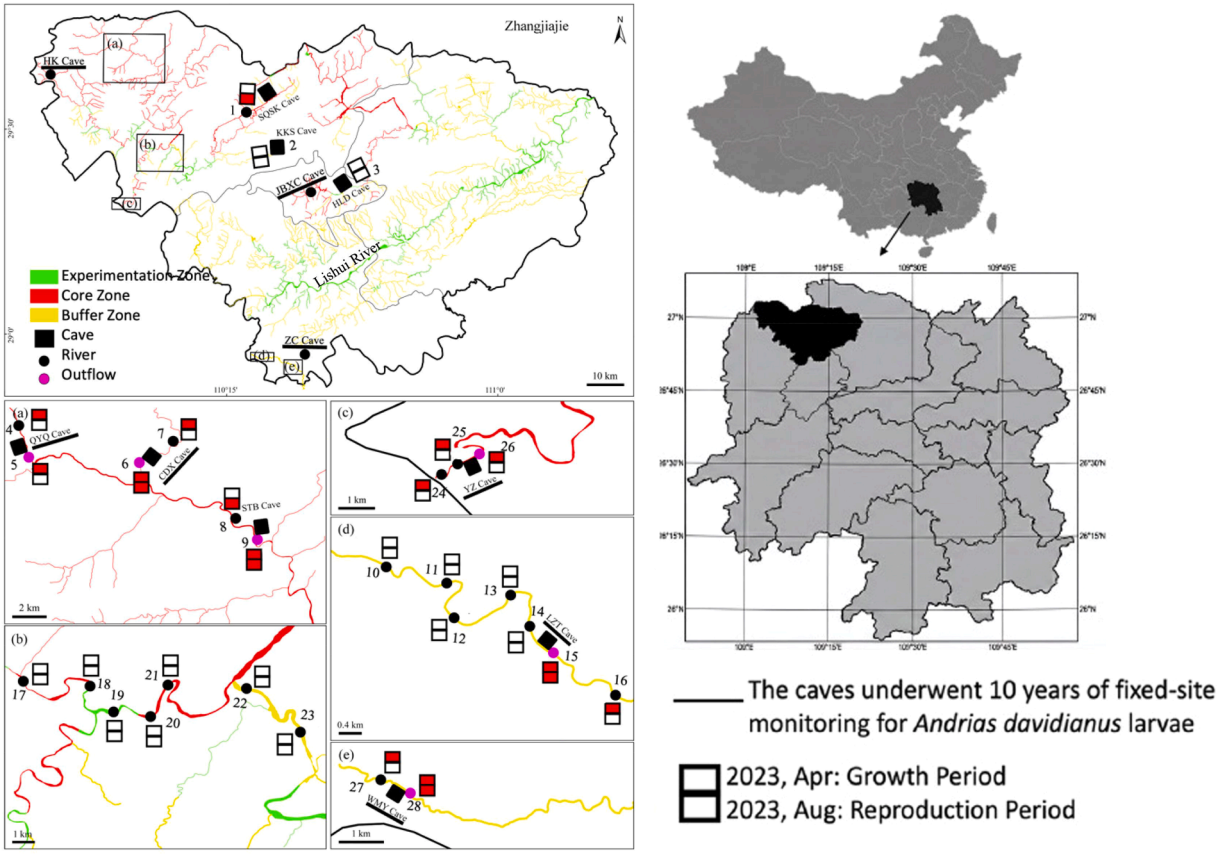

Fig. 1. Map of sampling sites near 11 natural caves in Zhangjiajie city (SQSK, KKS, JBXC, HLD, ZC, QYQ, CDX, STB, LZT, YZ, WMY Cave). Five caves (QYQ, CDX, STB, LZT, YZ) underwent 10 years of fixed-site monitoring for CGS larvae. Rectangles represent eDNA detection results from cave outflow and adjacent river sections in April 2023 (upper) and August 2023 (lower). Red indicates positive eDNA detection; white indicates negative.

2.1.2. Water Quality Traits of Habitat River Sections

To better comprehend the environmental features around the outflow caves of CGS larvae, this study mainly monitored the water quality of five caves (QYQ, STB, LZT, YZ, and WMY Cave) (Fig. 1; Appendix S2). In January 2013, environmental features were measured from river sections of these five caves (including outflow openings of each cave, and inflow openings of LZT and YZ Cave). Due to weather conditions, only outflow opening was measured from these five caves in January 2014 (Fig. 1; Appendix S2). At each sampling site, the following ten indicators were measured on-site: shading degree, substrate type, watershed, DO, water temperature, pH (SevenGo™pH-SG2, Mettler Toledo, Switzerland), geographical coordinates, altitude (Garmin 60CSX GPS, USA), flow velocity (LSH10–1A, China), and river width (measured with a tape measure).

2.1.3. Statistical Analysis

Drawing upon the aforementioned water quality characteristics, a regression analysis was conducted to examine the association between the water quality environment and the likelihood of emergence of CGS larvae. A linear model was employed for this investigation. In the process of model construction, the first step involved the establishment of the Full model, which encompassed the integration of all environmental variables. This methodological approach aimed to encompass all plausible predictor variables for comprehensive analysis. The operational procedures were structured into four distinct stages: (1) data preparation, (2) model formulation, (3) parameter estimation, and (4) model validation.

In the data preparation stage, all possible water quality parameters that might influence the emerging of CGS larvae were collected. Specifically, in this study, six environmental variables including shading degree, substrate type, watershed, DO, water temperature, and pH, were considered, and a Multiple Linear Regression (MLR) model was applied, as shown in Eq. 2:

$$y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \ldots + \beta_n X_n \quad (2)$$

where, y represents whether CGS larvae have emerged, while X_1, X_2, …, X_n refer to all the water quality environmental variables, and β_0, β_1, β_2, …, β_n are the coefficients to be estimated.

For the above model, the model parameters were estimated using Maximum Likelihood Estimation (MLE), which was accomplished through the lm() function in R. Model diagnosis was primarily based on the Akaike Information Criterion (AIC) value, with model performance assessed by comparing the AIC values across different models.

Theoretically, the construction of models encompassing all possible combinations of water quality parameters is a fundamental approach to determine the optimal model through the comparative analysis of their AIC values. Nevertheless, the sheer complexity of this endeavor necessitates the creation of a sizable number of models—26, to be precise—for a mere aggregation of six water quality parameters, thereby entailing an extensive computational burden. Hence, the present study has implemented the R programming language's advanced stepwise model selection function, known as stepAIC, to automate the process of identifying the optimal model. Stepwise regression, characterized by a deliberate interplay between model complexity and goodness of fit as determined by the AIC, executes a step-by-step iterative process of variable addition or elimination. Starting from a comprehensive full model, this method systematically eliminates variables one by one and re-evaluates the AIC. Should the AIC value exhibit a diminution upon the removal of a variable, said variable is discarded; conversely, it is retained if the AIC value remains unaltered. This iterative procedure continues until no further reduction in the AIC value is feasible through variable elimination, thereby culminating in the attainment of the optimal model configuration.

2.2. Experiment 2: Understanding the Distribution of Wild CGS through eDNA Technology

2.2.1. Collecting Water Samples

This research was conducted during the active phase of CGS. Previous research suggests a period of growth activity for the CGS in April, and the onset of its reproductive phase in August (Luo et al., 2021; Jiang, Tian and Zhang, 2022). Consequently, water samples were procured at two distinct temporal junctures: from March 31 to April 4, 2023, and from August 2 to August 10, 2023, resulting in a total of 54 samplings conducted across 27 designated sites encompassing the outlets of nine caves in addition to the proximate rivers (Fig. 1; Fig. S3). The water samples were obtained within a proximity of no more than 50 m from the cave outlets. A volume of 1 L of water sample was extracted from each sampling locale, with the immediate addition of 1 mL of Benzalkonium chloride solution (BCA) post-collection to forestall any potential sample deterioration (Yamanaka et al., 2017). Water sampling was conducted in triplicate at each site. Fresh gloves were employed for each sampling venture to ensure the integrity of the samples, adhering to the guidelines delineated by The Japan eDNA Society., (2019)). As part of the standard protocol, a field control sample was acquired daily, comprising two 500 mL bottles of mineral water, opened on site and augmented with 0.5 mL of BCA solution.

2.2.2. Water Sample Treatment and eDNA Extraction

All water samples were subjected to vacuum filtration using a glass-fiber filter with a nominal pore size of 0.7 μm (GF/F; Shanghai Bitai Biotechnology Co. Ltd., Shanghai, China) performed at the hotel on the day of sampling. The filter membranes were stored in 2 mL tubes (Shenggong, Shanghai, China) and frozen with dry ice for preservation. Upon returning to the laboratory, the samples were stored in a –20 °C refrigerator until DNA extraction. All filtration equipment was soaked in 10% hypochlorite solution for 5 min before and after use to remove residual DNA (Tian et al., 2024). After soaking, it was thoroughly rinsed with tap water and subsequently with commercial purified water (Hangzhou Wahaha Group Co. Ltd., China). Brand-new disposable gloves were used during filtration of each sample. Environmental DNA extraction was performed using the DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) following

the protocol of Wu and Minamoto (2023). To each sample, 440 μL of Buffer ATL and 40 μL of Proteinase K were added, followed by incubation at 56 °C in a water bath for 30 min and subsequent centrifugation at 15,000 × g. Subsequent DNA extraction followed the manufacturer's instructions, with final elution in 100 μL of AE Buffer (Qiagen, Hilden, Germany) prior to storage at –20 °C until PCR amplification.

2.2.3. PCR Amplification

PCR amplification was performed using the LightCycler 96 System (Roche Diagnostics) to amplify both field and field control samples, targeting the DNA information in the NADH1 region of CGS. Primers and probe developed in previous studies for the NADH1 region were used to detect DNA of CGS in water samples, without distinguishing differences in phylogeographic patterns (Fig. S4; Fukumoto et al., 2015). Each PCR amplification was conducted with three replicates per sample, along with three replicates of positive controls (DNA of the target species) and negative controls (pure water). Each TaqMan reaction mixture contained 900 nM primers, 125 nM probe, 1 ×gene expression premix (Life Technologies), and 2 μL of template DNA, with a total reaction volume of 20 μL. The qPCR reaction conditions were as follows: incubation at 50 °C for 2 min, denaturation at 95 °C for 10 min, followed by 55 cycles of 95 °C for 15 s and 55 °C for 60 s. Sample positivity was determined based on Ct values and amplification curves. A sample was considered positive if at least one replicate showed a positive signal (Wu et al., 2023). Furthermore, positive detections were confirmed by Sanger sequencing (Shanghai Sangon Biotech Co., Ltd., China).

3. Result

3.1. Experiment 1: Monitoring Natural Breeding Caves

3.1.1. The Outflow Time, Quantity Statistics, and Physiological Changes of CGS larvae

From 2005–2014, there were significant differences in the annual average outflow of CGS larvae across the nine natural breeding caves (Fig. 2; Fig. S1). Among them, YZ Cave had the highest annual average outflow (paired Wilcoxon rank sum test, $p < 0.05$), reaching 822 individuals per year, followed by CDX, QYQ, ZC, and HK Cave. STB Cave had the lowest annual average outflow, with only 15 individuals per year (Fig. 2; Fig. S1). Over this span of time, certain caves consistently demonstrated a pattern of "larval emergence" for consecutive years, with YZ Cave in particular showcasing this trend for a prolonged duration, excluding the year 2014. This underscores YZ Cave as displaying the lengthiest sustained period of larval emergence. In contrast, other caves experienced intermittent larval emergence (e.g., LZT Cave) or encountered interruptions in the occurrence of larvae during the final three years of the study period (Table S1).

In the course of this investigation, a notable discrepancy in the timing of larval emergence was observed between YZ and WMY Cave (Fig. 3). Specifically, the onset of larval emergence among CGS in YZ Cave commenced on approximately December 6, culminating around December 31. This duration encompassed a period of approximately 26 days (Fig. 3a, b). In contrast, the emergence of larvae in WMY Cave occurred notably later, typically commencing around January 20 and concluding around February 10, spanning a period of approximately 20 days (Fig. 3c–e).

Additionally, there was a noticeable difference in the outflow rate of CGS larvae between the two caves on the same day, both of which exhibited notable diurnal variations. The outflow rates of CGS larvae in YZ and WMY Caves were found to be most rapid during the first half night (19:00–23:00), with YZ Cave reaching its highest "larval emergence" speed in 2012, at 6.78 individuals per hour (Table 1). During the daylight hours (7:00–19:00), the outflow rate was slowest, and in WMY Cave, no larvae were observed to flow out during this period in 2011 and 2013. The outflow rate was lowest during the early morning hours (3:00–7:00), while the rate was higher during the late-night period (23:00–3:00).

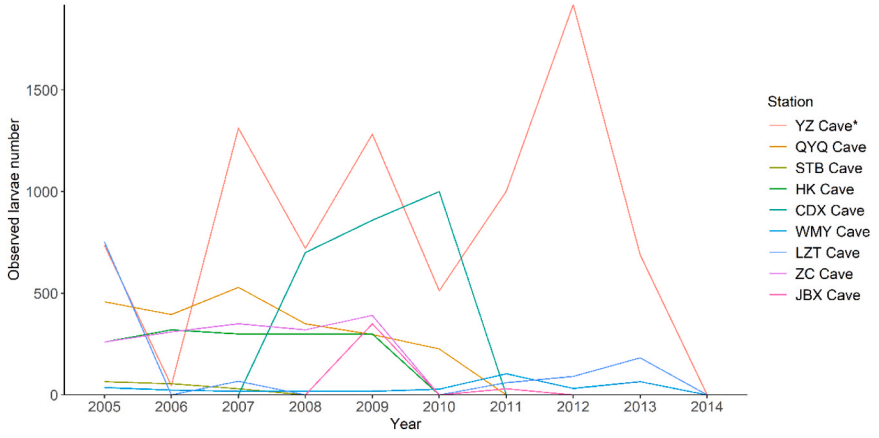

Fig. 2. Temporal changes in CGS larvae outflow from nine caves in Zhangjiajie City in China (2005–2014). Pairwise Wilcoxon rank sum tests were conducted with Holm-Bonferroni correction to adjust for multiple testing, with independence assumption based on the result (*$p < 0.05$).

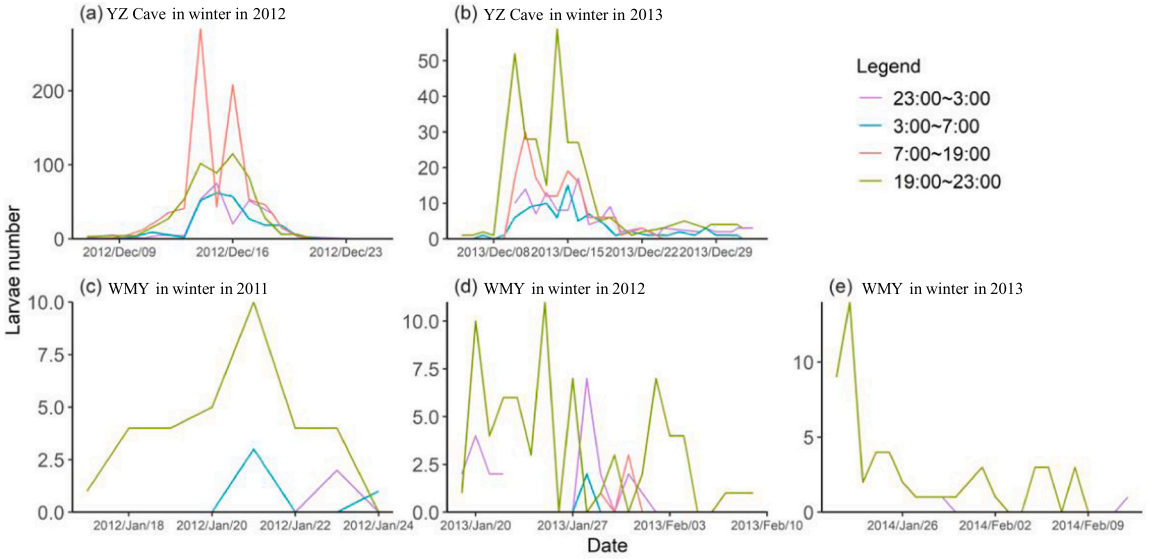

Fig. 3. Quantity of CGS larvae outflowing from YZ (2012, 2013) and WMY (2011–2013) Caves in winter.

Table 1
Velocity (individuals/hour) of larvae outflowing from two caves in Zhangjiajie City in China from 2011 to 2013.

Location	Cave Name	Date of larvae outflowing	23:00–3:00	3:00–7:00	7:00–11:00	11:00–15:00	15:00–19:00	19:00–23:00
Sangzhi County	YZ Cave	Dec. 8–Dec. 27, 2012	3.53	3.33	3.45	2.01	4.14	6.78
	YZ Cave	Dec. 6, 2013-Jan.2, 2014	1.13	0.81	0.46	0.21	0.63	2.90
Yongding District	WMY Cave	Jan.18–Jan.25, 2012	0.06	0.13	0.00	0.00	0.00	1.00
	WMY Cave	Jan.20–Feb.11, 2013	0.25	0.04	0.00	0.00	0.01	0.78
	WMY Cave	Jan.22–Feb.13, 2014	0.07	0.07	0.00	0.00	0.00	0.58

In terms of the body color change progress of CGS larvae after leaving the cave, it was observed that the larval skin was initially flesh-colored when they first emerged (Fig. 4). After being exposed to external light, their body color gradually changed from flesh-colored to dark black, with the sequence of body color darkening as follows: tail, back, and belly (Fig. 4). Furthermore, the speed of body color change in CGS larvae from YZ Cave was negatively correlated with the intensity of external light. The higher the light intensity, the faster the body change, and the shorter the time required to complete the darkening process. Under the light conditions of 100–150 lux, the tail turned black in 4 h and the entire body in 6 h. While under the light intensity of 10–20 lux, these times were 7 and 12 h, respectively (Table 2).

3.1.2. Water Quality Traits of Habitat River Sections

Table 3 shows the AIC values for different models and the AIC differences (ΔAIC) relative to the best model. AIC is an index used to compare the goodness of fit of statistical models, where a lower AIC value indicates a better model. Therefore, the optimal model identified was the one comprising "shading degree + river width + flow velocity + DO," with the AIC value of –23.59 and ΔAIC of 0 (set as the reference benchmark). Other models were ranked in ascending order of AIC values as follows: "shading degree + river width + flow velocity + DO + pH," "river width + flow velocity + DO," "shading degree + river width + flow velocity," and "shading degree + river width + flow velocity + DO + water temperature." In general, models with $\Delta AIC < 2$ are deemed statistically non-significantly different from the optimal model and possess strong explanatory power (Wu et al., 2023). Thus, the disparities among the examined models herein do not exhibit substantial significance.

By examining the components of each model, it can be found that: (1) "river width + flow velocity + DO" is the most frequently occurring variable combination, (2) the incorporation of additional variables such as "pH" or "water temperature" does not yield a significant improvement in model performance, and (3) the simplest yet effective model appears to be the one containing solely "river width + flow velocity + DO", which, despite consisting of only three variables, demonstrates a minimal increase (0.62) in AIC compared to the optimal model. In conclusion, the model featuring "shading degree + river width + flow velocity + DO" is the superior choice. Nonetheless, the model comprising "river width + flow velocity + DO" also presents itself as a commendable alternative due to its succinct nature and competitive performance.

3.2. Experiment 2: Understanding the Distribution of Wild CGS through eDNA Technology

During two survey periods, 18 of 58 samples collected from seven caves (excluding KKS Cave, HLD Cave, and Region C) tested

Fig. 4. Body coloration of *Andrias davidianus* larvae. Wild larvae from the YZ cave, showing time elapsed since first leaving the cave: 1, 15 h; 2, 5 h; 3, 20 min; 4, 50 min; 5, 50 min; 6, 10 min; 7, 12 h. Photo from Liang et al., (2019).

Table 2
Time to full black coloration in CGS larvae after leaving YZ cave, under different light conditions ($n = 8$).

Illumination Intensity (lux)	Time of the tail finished to be entirely black (h)	Time of the body finished to be entirely black (h)
100–150	4.45 ± 0.65^{a}	6.24 ± 0.97^{a}
50–80	6.41 ± 0.76^{c}	9.21 ± 0.61^{c}
10–20	7.35 ± 0.71^{d}	12.11 ± 1.24^{e}

Note: Values with neighboring superscripts in the same column indicate significant difference ($P < 0.05$). Values with apart superscripts in the same column donate extremely significant difference ($P < 0.01$).

positive for CGS DNA (Fig. 1; Fig. S3). Positive PCR amplicons were sequenced and confirmed to match the target species. Among these, target DNA was detected at four cave outlets (Fig. 1). During March 31–April 4, 2023, DNA was detected not only at outlets of QYQ, CDX, YZ, and WMY Cave but also in surrounding rivers (Fig. 1). During the August 2–10, 2023 survey, 6 of 29 sampling sites tested positive for CGS DNA (Fig. 1). Notably at STB Cave, DNA was detected both at the outlet and in surrounding rivers (Fig. 1). No amplification of target species DNA was observed in either field blank controls or PCR negative controls.

4. Discussion

Through long-term monitoring of CGS in natural breeding caves across China, this study documented continuous larval emergence in certain caves over multiple years, with spatiotemporal variation in emergence timing. Additionally, the study recorded the complete

Table 3
The influence of water quality on the presence of outflow larvae was assessed using linear models. Model selection was performed by comparing AIC values across various model combinations to identify the optimal model.

Model	AIC	ΔAIC
shading degree + river width + flow velocity + DO	–23.59	0
shading degree + river width + flow velocity + DO + pH	–22.98	0.61
river width + flow velocity + DO	–22.97	0.62
shading degree + river width + flow velocity	–22.79	0.8
shading degree + river width + flow velocity + DO + water temperature	–22.76	0.83
shading degree + river width + flow velocity + water temperature + pH	–22.23	1.36
river width + flow velocity + DO + pH	–22.01	1.58
shading degree + river width + flow velocity + DO + water temperature + pH	–21.76	1.83
shading degree + river width + flow velocity + pH	–21.35	2.24
river width + flow velocity + DO + water temperature + pH	–21.16	2.43
shading degree + river width + DO	–17.96	5.63
shading degree + river width + DO + pH	–16.65	6.94
shading degree + river width + DO + water temperature + pH	–16.27	7.32
shading degree + flow velocity + DO	–14.04	9.55
shading degree + flow velocity + DO + water temperature + pH	–12.65	10.94
shading degree + flow velocity + DO + pH	–12.06	11.53

post-emergence body color change process in larvae and characterized breeding cave environments. Environmental DNA analysis further revealed persistent wild individuals in Zhangjiajie city, suggesting that caves without recent larval observations may still harbor adult individuals. These results provide critical data support for future ecological research and conservation management of wild CGS, aiding the development of effective protection measures to ensure the species' long-term persistence.

4.1. Larval Emergence Patterns and Associated Ecological Features in CGS

This study analyzed larval emergence patterns across caves, associated ecological features, and long-term trends. Significant inter-cave variation was observed (Fig. 2; Fig. S1). YZ Cave exhibited the highest mean annual emergence (822 individuals/year), while STB Cave showed the lowest (15 individuals/year) (Fig. S1). Environmental monitoring identified shading at cave entrances, water flow velocity, DO, and river width as primary factors influencing emergence. Water temperature and pH also affected emergence probability (Table 3). CGS require pristine aquatic habitats (Liang et al., 2004; Browne et al., 2013) with abundant food resources (Wang et al., 2004). Their natural habitats—karst caves, deep pools, and subterranean rivers—provide stable thermal regimes, low flow velocities, and high shading, offering ideal refuge (Luo and Kang, 2009; Tao, Wang and Zhang, 2004). Reef and pebble-dominated substrates enhance concealment while maintaining water quality and oxygenation (Wahl, 2009; Kingma et al., 2024). Riparian vegetation stabilizes habitats, reduces erosion, provides shade, and moderates thermal fluctuations (Davis et al., 1996). Cave microhabitat characteristics likely reflect larval physiological requirements and behavioral adaptations. Shading offers predator refuge while indirectly influencing food resource availability through light modulation (Hayes, Stark and Shearer, 1994). Water flow velocity directly affects larval habitat selection, and excessively high or low water flow velocities may prevent them from foraging effectively or escaping predators (Zhang et al., 2006). Water temperature, however, significantly impacts larval survival by affecting metabolic rates and development speed, as larvae of many species are extremely sensitive to temperature changes (Yang et al., 1983). In particular, the first winter is the most critical period, exerting the greatest impact on the survival of larvae. Furthermore, water quality (e.g., DO and pH) is crucial for larval survival, while low DO concentration can significantly affect embryonic development (Liu, Xiao and Yang, 1995). These factors collectively demonstrate that the survival of aquatic larvae often depends on specific habitat conditions. For example, larvae of freshwater invertebrates such as Ephemeropteras show high sensitivity to water quality and flow velocity (Brittain and Saltveit, 1989), while the distribution in rivers of vertebrate larvae such as *Oncorhynchus* spp. babies is closely related to water flow velocity, substrate type, and water temperature (Bjornn and Reiser, 1991).

In caves exhibiting intermittent or ceased larval emergence, reduced reproductive output primarily stems from parental senescence and diminished embryonic survival. Aging breeders exhibit impaired reproductive capacity, including reduced fecundity, poorer egg quality, and lower fertilization rates (Browne et al., 2002). As long-lived amphibians, CGS experience significant reproductive decline with advancing age (Liang et al., 2004). Embryonic survival is further compromised by multiple factors, including water temperature, DO concentration, water quality, pathogen infection, and so on (Blaustein et al., 2011). Extreme water temperatures disrupt embryonic development, causing hatching failure or teratogenesis (Yang et al., 1983), while pollutants in water (such as heavy metals and pesticides) may impact egg development through permeation (Luo et al., 2009b). Habitat degradation (e.g., hydropower station construction and agricultural activities) may further exacerbate these issues, disrupting the breeding environment of CGS and reducing their egg survival rates (Wang et al., 2004).

Consequently, effective conservation strategies for CGS must address both environmental drivers of larval survival and adult reproductive capacity. Habitat quality enhancement through riparian vegetation restoration, aquatic system maintenance, and pollutant discharge control will enhance embryonic survival, while targeted protection of breeding adults is critical for individual viability. These integrated measures promote sustainable recovery of wild individuals.

Environmental parameters, including river shading degree, river width, flow velocity and DO are critical factors influencing the

conservation of the CGS. Maintaining river connectivity and implementing appropriate spatial planning for human activities in Zhangjiajie are essential to ensure unobstructed migration across life stages—such as breeding, foraging, and dispersal—thereby reducing the risk of population isolation. Furthermore, the watershed should be managed as an integrated ecological unit rather than as fragmented river sections. Given that Zhangjiajie is a globally renowned tourist destination, it is imperative to balance tourism development with CGS conservation. This requires the strategic designation of tourist zones to prevent anthropogenic disturbances from degrading critical habitats or interfering with CGS biological activities.

4.2. Timing Differences of Larval Emergence and Day-night Rhythms of CGS Between Caves

Larval emergence in CGS exhibits significant inter-cave variation and distinct diel rhythms. Intensive monitoring revealed divergent phenology: emergence in YZ Cave peaked in December, whereas in WMY Cave, it occurred primarily from January to February of the following year (Fig. 3). Because CGS larvae rely on yolk sacs for nutrition for approximately 40 days post-hatching (Fan et al., 2022), their age was estimated by comparing wild yolk sac absorption and body length with developmental data from artificially propagated individuals (Zhang, 2009; Fan et al., 2022). Consequently, larvae from YZ and WMY Caves were estimated to be approximately 45 and 55 days old, respectively, at the time of emergence. Integrating these estimates with cave water temperature data, natural hatching periods for both sites were inferred to be approximately 45 days, suggesting that mating likely commenced in early September. Temperature differences are known to influence amphibian reproductive timing (Liang et al., 2004). The significantly lower water temperature in WMY Cave compared to YZ Cave (Fig. S2) potentially delayed larval hatching. Microhabitat conditions—including flow velocity, DO concentration, and nutrient availability—may further influence developmental processes in CGS larvae, contributing to emergence timing differences. Crucially, giant salamanders in YZ and WMY Caves represent distinct population (Liang et al., 2019), suggesting reproductive divergence between lineages may additionally drive timing variations.

Moreover, the pattern of larval emergence observed in both caves displayed a notable day-night rhythm. Peak emergence periods for YZ and WMY Caves occurred before midnight (19:00–23:00), with minimal emergence observed during daytime (07:00–19:00) (Fig. 3; Table 1). Notably, in some years at WMY Cave, no larval emergence occurred during daylight hours. Such nocturnal activity pattern is observed in some amphibians that forage mainly at night (e.g., *Pelophylax nigromaculatus*, *P. plancyi*, and *Bufo gargarizans*), particularly in warm and humid seasons (Fei and Ye, 2025). This behavior trait is closely related to their physiological structure, nutritional requirements, and predator avoidance strategies (Wells, 2007; Fei and Ye, 2025). In this study, the observed pattern likely reflects the species' nocturnal nature, serving to mitigate predation risk and avoid physiological stress associated with intense light exposure. The emergence frequency peaked between 19:00–23:00 (Table 1), suggesting the early night provides optimal migration conditions. These patterns provide important support for formulating monitoring plan against wild CGS in the future, that is, night surveys (particularly 19:00–23:00) more effectively capture larval emergence dynamics.

The YZ and WMY CGS individuals belong to two distinct genetic lineages, respectively (Liang et al., 2019). Outflow time of YZ CGS larvae from caves was significantly earlier than that of WMY, indicating that natural mating times of two populations were also different, and there may be differences in their reproductive ecological requirements. Therefore, protecting the biodiversity of giant salamanders requires not only protecting genetic diversity but also protecting ecological diversity. Specific protection strategies should be formulated for YZ population and WMY population, respectively.

4.3. The Ecological Significance of Body Color Changes in CGS Larvae

Through tracking body color changes in CGS larvae after emergence from caves, we discovered an absence of melanin in their skin upon initial emergence. Following light exposure, rapid melanin synthesis occurs, with pigmentation persisting permanently during growth (Fig. 4). This indicates natural spawning and hatching in Zhangjiajie occur exclusively in dark cave environments. Our results reveal a negative correlation between post-emergence darkening rate and light intensity: higher light intensity accelerates darkening. Larvae darken sequentially in tail, back, and belly parts (Fig. 4), reflecting pigment cell stress responses to light. Under high illumination, accelerated melanin synthesis may enhance UV protection and reduce light damage, consistent with observed larval characteristics. Due to long-term exposure to dim conditions in the cave environment, the skin of CGS larvae presents a relatively light flesh color. After flowing out from the cave, the CGS larvae exhibit a striking transformation in their body coloration. Their once dark hue now serves as a form of natural camouflage in the external environment, potentially minimizing the threat of predation. This adaptive mechanism, unique to these elusive creatures, hints at a complex evolution of survival strategies in the wild. The phenomenon of color change among juvenile salamanders highlights their remarkable ability to adjust to the challenges of their newfound surroundings. This tactic of blending into their environment to evade predators is not uncommon in the animal kingdom, with similar behaviors observed in a variety of species (e.g., Frogs of the *Hyla* genus; Choi and Jang, 2014; Kang, Kim and Jang, 2016).

4.4. The Indicative Role of DNA Test Results on the Distribution of CGS

By utilizing eDNA technology, this study conducted a systematic investigation into the habitat distribution of CGS in Zhangjiajie city of Hunan Province. The findings revealed that CGS DNA was detected not only in caves characterized by year-round larval emergence but also in those where emergence had become intermittent or had ostensibly ceased in recent years (Fig. 1; Fig. S3). These results demonstrate the existence of a sizeable individual of wild CGS in Zhangjiajie city. During periods of peak activity for CGS, eDNA was detected in four caves (CDX, STB, LZT, WMY) during two distinct investigation periods (Fig. 1; Fig. S3), indicating that these caves could serve as stable habitats for CGS due to favorable environmental conditions that support their long-term survival. Moreover, CGS

DNA was not only detected at the water outlets of QYQ, CDX, YZ, and WMY Caves but also in the rivers surrounding them (Fig. 1; Fig. S3). This suggests that these caves may represent key habitats for CGS, with their activity potentially extending into the surrounding waters. This discovery lends further support to the notion that the stable environmental conditions around these caves create an ideal habitat for CGS (see discussion in Section 4.1.).

Critically, CGS DNA persists in caves experiencing intermittent or ceased larval emergence, confirming remnant individuals inhabit these sites. These findings align with 2022 survey data from Zhangjiajie National Nature Reserve (Zhou et al., 2024), confirming methodological reliability in this study. However, the presence of CGS DNA in these cases without corresponding larval emergence may suggest senescent breeders and reduced embryonic viability (see discussion in Section 4.1.). Additionally, this phenomenon could be linked to nutritional constraints within wild cave habitats, which may extend the reproductive cycle from annual to biennial, particularly in females. To gain a deeper understanding of the reproductive ecology of CGS in these caves, it is recommended that future research incorporates long-term DNA monitoring along with the analysis of the ratio between nuclear and mitochondrial genes (Wu et al., 2022). This approach allows tracking the reproductive dynamics of CGS in these caves and determining whether they retain reproductive capability. Liang et al. (2019) identified multiple populations in the CGS. Admittedly, eDNA technology possesses inherent limitations; specifically, it currently lacks the resolution to differentiate between life-history stages (e.g., adults vs. larvae) or to distinguish among the distinct genetic lineages of the CGS. Furthermore, the reliance on short target fragments may lead to an overestimation of occupancy due to their elevated environmental persistence. To address these constraints, future research should integrate environmental RNA technology (Littlefair, Rennie and Cristescu, 2022; Wang et al., 2025) or utilize longer DNA fragments (Jo and Yamanaka, 2022) to develop stage-specific markers. Additionally, prioritizing the development of lineage-specific primers will be essential to accurately delineate the geographic distribution of these distinct populations.

4.5. Research Significance and Conservation Suggestions

This study, employing long-term field monitoring and molecular techniques, uncovered the reproductive ecological features, day-night activity rhythms, and habitat distribution of wild CGS in Zhangjiajie city. These findings lay a crucial scientific foundation for the conservation of CGS. Building on the research results, the following conservation suggestions are put forward:

1) Strengthen Protection of Key breeding habitat: YZ Cave stands out with the highest average annual larval emergence frequency, making it a top priority area for safeguarding the reproduction of CGS. Human activities should be minimized to preserve a suitable habitat environment.
2) Optimize Field Survey Protocols for CGS: Based on day-night rhythm data, future relevant field monitoring against CGS should prioritize nighttime observations (19:00–23:00) to improve survey efficiency.
3) Continuously Monitor Changes of Habitat Environment: The cessation of larval emergence in some caves signals the need for a more thorough evaluation of ecological changes impacting CGS before implementing necessary restoration measures, such as improving water quality or supplementing food resources.
4) Expand the Scope of DNA Monitoring: This study identified traces of CGS in nearby river systems surrounding some karst caves. Therefore, DNA monitoring can be carried out in a broader river basin in the future, to comprehensively evaluate the distribution and habitat utilization of CGS.

CRediT authorship contribution statement

Zhiqiang Liang: Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Funding acquisition, Conceptualization. **Mingqiu Liu:** Investigation. **Qiwei Wei:** Supervision, Methodology, Conceptualization. **Chongrui Wang:** Investigation. **Xunhua Liu:** Investigation. **Xuan Xie:** Investigation. **Linmei Han:** Investigation. **Zhiyong Deng:** Investigation. **Jinxin Zhou:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Data curation, Conceptualization. **Qianqian Wu:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Methodology, Investigation, Data curation, Conceptualization. **Li Zou:** Investigation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.gecco.2026.e04186.

Data availability

All raw data for this study have been compiled in the Appendix.

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