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Comparative morphometric analysis of erythrocytes among several reptile species using ImageJ

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Abstract

Background The morphometry of erythrocytes is highly correlated to its metabolic rate and metabolic demand of the species, which cause the main difference between reptiles and mammals. Reptiles are ectotherms which has very low metabolic rate, but has variation to its metabolic demand due to the differences between its phylogenetic, habitat, and behavior.

Objective The aim of the present study was to compare erythrocyte morphometry among several reptile species.

Methods Blood smear samples that were obtained from published articles and analysed using ImageJ software. Surface area and circularity of erythrocyte and its nucleus were analyzed, and the surface area ratio between erythrocyte and nucleus was calculated.

Results Sea turtle that spends most of its life in the sea has the smallest erythrocyte (84.04 ± 7.24 μm^2) due to its behavior to dive and requires more rapid and effective blood gas exchange. Meanwhile, terrestrial reptiles that have passive behavior, such as Boa snakes, has the largest erythrocyte (270.59 ± 10.12 μm^2) which allows slow circulatory rate but can also carry more gas during activity.

Conclusion This comparative study showed that not only phylogenetic characteristic of a species, but habitat and behavior that affects metabolic rate and blood gas exchange are also reflected in the morphometry of erythrocytes.

Keywords Erythrocyte | ImageJ | metabolic rate | morphometry | reptile

Introduction

Blood cell morphology varies among vertebrate and invertebrate animals. Variation in erythrocyte morphology includes shape, nucleus, and size of the cell. The morphology usually differs related to its habitat and physiological processes. Morphology variation of erythrocyte cells related to their hemoglobin content and surface area may influence their main role in transporting oxygen and carbon dioxide throughout the body (Hawkey *et al.,* 1991). Generally, factors that may affect the morphology of erythrocytes are body size, metabolism rate, and body temperature of the animal.

Among vertebrates, birds, fish, amphibians, and reptiles have elongated and nucleated erythrocytes. Hemoglobin of these animals is located in the nucleus, which allows mitotic and amitotic division of erythrocytes after cell maturation (Bodes & Martinez, 2023). Immature erythrocytes with mitotic activity are usually found in the peripheral blood of reptiles. Immature and mature erythrocytes both have different shapes and sizes. Small, irregularly rounded shapes with large nuclei are immature erythrocytes, while the mature cells are larger than the immature cells and have smaller nuclei (Campbell, 2012).

According to Hawkey *et al.* (1991), variation of erythrocyte sizes is closely related to their hemoglobin content; larger

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erythrocytes contain a higher concentration of hemoglobin and vice versa. Vertebrates with a smaller size of erythrocyte will require more circulating erythrocytes to fulfill the physiological tissue oxygen demand. This pattern was shown in the comparison of physiological sizes of erythrocytes among vertebrates, where reptiles have the largest size of erythrocytes along with a higher concentration of hemoglobin, but has fewer total erythrocyte count compared to mammals and birds (Hawkey *et al.,* 1991). On the other hand, larger cells have a smaller surface area relative to their volume, which slows the rate of gas diffusion across the cell membrane. High concentration of hemoglobin in large-sized erythrocytes could also act as compensation, as larger cells will move through capillaries less efficiently and have a lower rate of gas exchange.

The size of erythrocytes is species-specific, which is formed through the evolution of the animal. A morphometric comparison of erythrocytes in rodents by Kizhina *et al.* (2020) showed that erythrocyte size tends to increase with the increasing body mass of an animal, although this pattern was not consistent across different taxonomic levels. Morphometrical and blood profile variation of the erythrocytes could be affected by the habitat (Janecka *et al.*, 2015) and behavioral differences (Udroiu, 2023) across the species. Differences in habitat (depth and elevations, low or high environmental temperature) lead to animals' adaptation by adjusting their physiological traits, including oxygen affinity of erythrocytes and their metabolic rate (Udroiu, 2023). A study by Udroiu (2023) showed that small erythrocytes allow rapid oxygen release in tissues with high metabolic demand, and the size differences of erythrocytes across species could be viewed as an adaptation to adjust the oxygen affinity in the erythrocyte. Semiaquatic rodents such as capybara, beaver, and nutria have larger erythrocytes due to their habitat, since animals that swim and dive frequently require greater oxygen capacity (Kizhina *et al.*, 2020).

Comparative studies on erythrocyte morphometrics in mammals and birds have been conducted widely, yet remain limited in reptiles. Given their wide ecological diversity, poikilothermic physiology, and evolutionary divergence, reptiles provide an interesting model to explore how environmental, physiological, and phylogenetic factors influence erythrocyte morphometry. Variation in erythrocyte and nuclear size among reptile taxa may reflect the adaptive responses to ecological conditions and inherited phylogenetic traits. This study aims to analyze and compare erythrocyte morphometrics across several reptile species using ImageJ to provide insight into the relationship between cell morphology, ecology, and evolutionary background.

Methods

Sample Collection

Blood smear sample images were obtained from published articles from open-access journals or official websites using a purposive sampling method. The selected samples were blood smear images of healthy adult reptiles under normal physiological conditions and natural habitat. The number of individuals represented per species varied depending on the availability of published sources. Criteria that were used during sample collection are: the presence of a measurement scale bar, a proper staining quality allowing clear visualization of erythrocyte and nuclear boundaries, and minimally overlapping cells with distinguishable margins. The number of individual animals represented per species depends on the published sources. However, in several articles, the exact number of individual animals contributing to the blood smear image was not explicitly reported. Therefore, the exact number of individuals represented in several species could not be determined.

Different article sources may give a different staining technique, smear preparation, fixation protocols, microscope type, magnification, and image resolution. To minimize any potential measurement bias, all images were digitally calibrated using the original scale bar provided in each figure. Images were enhanced using Adobe Photoshop 2021 to improve clarity and contrast without altering cell morphology. At least 6 erythrocyte cells per species were measured using ImageJ software.

Erythrocyte Morphometrical Analysis

The figures were converted into 8-bit grayscale images, and calibration was performed according to the measurement bar to convert pixels into micrometers (μm). The image will be adjusted to select the area of the particles measured. Cells that were clustered or slightly overlapped will be separated using the binary-watershed feature on ImageJ. Parameters analyzed were the surface area (μm^2) and circularity (0–1) of erythrocytes and their nuclei. Circularity is a measure of how round the particle or cell is compared to a perfect circle. A circularity value of 1.0 indicates a perfect circle, while a lower value indicates increasing elongation. The ratio between cell area and the nucleus area is also measured as one of the parameters. Although digital calibration was applied to minimize measurement variability, differences in staining contrast, smear thickness, fixation-induced shrinkage, and imaging resolution across different sources may influence absolute morphometric values. Therefore, interpretations were made cautiously with emphasis on relative comparisons rather than absolute measurements.

Data Analysis

The collected data will be analyzed using IBM SPSS 26 to obtain descriptive statistics and to perform comparative tests among different species. Statistical analysis was performed using individual erythrocyte measurements as the analytical unit (**Table 1**). Individual-level replication could not be verified since the number of individuals represented in several source images was not always reported. Data distribution will be assessed for normality and homogeneity using the Shapiro-Wilk test with the assumption of normality ($P>0.05$). If the data are normally distributed, a one-way ANOVA test will be conducted. If the data are not normally distributed, an appropriate data transformation would be applied. If normality cannot be achieved, a non-parametric analytic Kruskal-Wallis test will be conducted. Post-hoc analysis using Tukey's test will be performed when significant differences are observed ($P<0.05$).

Table 1 Number of samples acquired for statistical analysis

Species	*n* (cells measured)	*n* (Individuals, if reported)	Reference
Green sea turtle (*Chelonia mydas)*	12	Not reported in original source	Stacy & Boylan, 2014
Santa Cruz Galapagos tortoise (*Chelonoidis porteri)*	13	210	Nieto-Claudín *et al.,* 2021
Flowerback box tortoise (*Cuora galbinifrons)*	10	Not reported in original source	Stacy *et al.,* 2011
Green iguana (*Iguana iguana)*	21	Not reported in original source	Stacy *et al.,* 2011
Egyptian Nile monitor lizard (*Varanus niloticus)*	20	6	Soliman *et al.,* 2023
Eurasian blind snake (*Xeroptyphlops vermicularis)*	11	Not reported in original source	Arikan *et al.,* 2009
Dahl's whip snake (*Platyceps najadum)*	7	Not reported in original source	Arikan *et al.,* 2009
Common boa snake (*Boa constrictor imperator)*	6	Not reported in original sources	Stacy *et al.,* 2011

Results

The measurement of erythrocytes and the nucleus size of several reptiles are shown in **Table 2**. The parameters measured include cell and nuclear areas, circularity, and the erythrocyte-to-nucleus area ratio. The erythrocyte area varied significantly among species ($P<0.05$). The largest erythrocyte was observed in the common boa constrictor (*Boa constrictor*) (270.59 ± 10.12 μm^2), while the smallest belongs to the green sea turtle (*Chelonia mydas*) (84.04 ± 7.24 μm^2) (**Figure 1**). Iguana (*Iguana iguana*) had a larger surface area (193.78 ± 19.22 μm^2) than that of Egyptian Nile monitor lizard (*Varanus niloticus*) (108.48±15.85 μm^2). Both Eurasian blind snake (*Xeroptyphlops vermicularis*) and Dahl's whip snake (*Platyceps najadum*) showed no significant differences in surface area (121.74 ± 12.52, 123.53 ± 10.91 μm^2). Erythrocytes circularity ranged from 0.76 to 0.86, indicating they were elongated and round. *Platyceps najadum* had the highest value of erythrocytes circularity (0.86 ± 0.01), indicating a more circular cell shape than the other species. Ratio of erythrocyte-to-nucleus area varied among species, with the *C. galbinifrons* turtle exhibiting the highest ratio (8.09 ±1.03). This indicates that this species had larger erythrocyte cytoplasm (the cell is much larger than the nucleus) (**Figure 2**).

Table 2 Erythrocytes morphometry in several reptiles

Species	Erythrocytes		Nuclei		Ratio area of erythrocyte/ nuclei
	Surface Area (μm^2)	Circularity	Surface Area (μm^2)	Circularity	
Chelonia mydas	84.04 ± 7.24^{a}	0.84 ± 0.02^{bc}	11.15 ± 0.67^{a}	0.86 ±0.05^{de}	7.56 ± 0.63^{cd}
Chelonoidis porteri	114.47 ± 10.83^{b}	0.80 ± 0.03^{a}	20.71 ± 1.15^{b}	0.89 ±0.03^{e}	5.52 ± 0.49^{a}
Cuora galbinifrons	155.23 ± 8.12^{c}	0.77 ± 0.04^{a}	19.46 ± 2.36^{b}	0.82 ± 0.05^{cd}	8.09 ± 1.03^{d}
Iguana iguana	193.78 ± 19.22^{d}	0.76 ± 0.04^{a}	34.02 ± 2.98^{c}	0.81 ± 0.04^{c}	5.72 ± 0.59^{a}
Varanus niloticus	108.48 ± 15.85^{b}	0.76 ± 0.03^{a}	18.93 ± 1.42^{b}	0.81 ± 0.04^{cd}	5.74 ± 0.87^{ab}
Xeroptyphlops vermicularis	123.53 ± 10.91^{b}	0.79 ± 0.04^{a}	21.42 ± 2.07^{b}	0.76 ± 0.04^{bc}	5.79 ± 0.53^{ab}
Platyceps najadum	121.74 ± 12.52^{b}	0.86 ± 0.01^{c}	20.83 ± 1.68^{b}	0.71 ± 0.11^{b}	5.84 ± 0.49^{ab}
Boa constrictor	270.59 ± 10.12^{e}	0.80 ± 0.03^{ab}	40.71 ± 4.4^{d}	0.61 ± 0.08^{a}	6.72 ± 0.67^{bc}

Note: Different superscripted letters show significant differences ($P<0.05$)

Discussion

According to the morphometric analysis results, there are significant differences among different species and families. Reptiles, as ectothermic animals, generally have a much lower metabolic rate compared to mammals, which are endothermic (Nagy, 2005). The metabolic rate of a reptile correlates with the body size, habitat or climatic condition, its natural behavior (fast or slow pace of life), and its response to climatic change (Nagy, 2005; Dupoué *et al.*, 2017; Carter *et al.*, 2023). Ectotherms mainly rely on external heat sources to regulate body temperature, so their overall energy expenditure is lower and so is their oxygen demand. According to Hawkey *et al.* (1991), erythrocyte size has a positive association with hemoglobin content, and thus affects the rate of blood cell circulation for gas exchange. The rate at which gas

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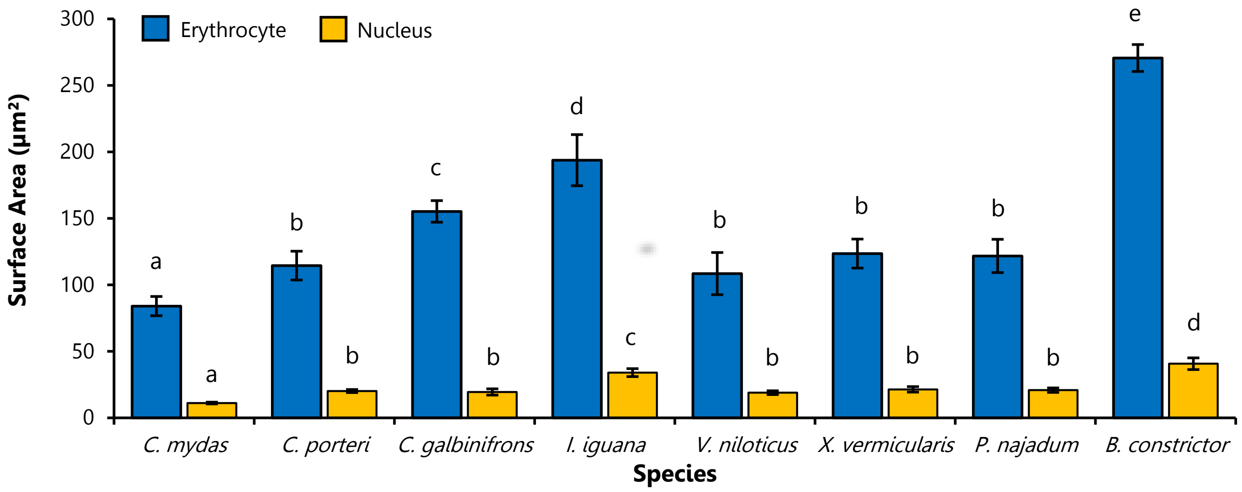

Figure 1 Erythrocyte and nucleus surface areas comparison between reptile species. Our findings show that *Boa constrictor* has the largest erythrocyte surface area (270.59 ± 10.12 μm^2), while *Chelonia mydas* has the smallest erythrocyte surface area (84.04 ± 7.24 μm^2). Different letters indicate significant differences ($P<0.05$).

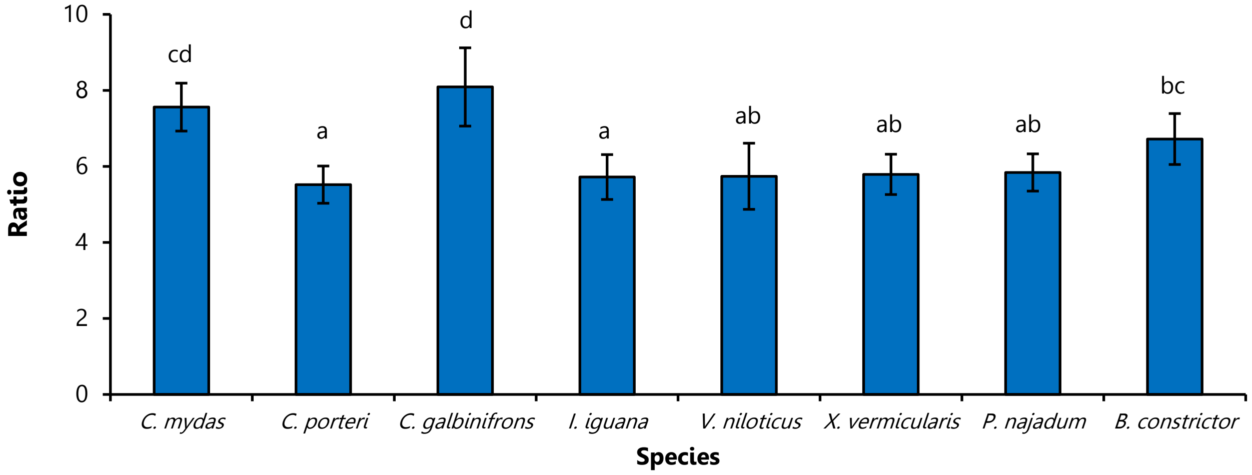

Figure 2 Erythrocyte to nuclei ratio comparison between reptile species. In the present study, *Cuora galbiniformis* has the largest ratio (8.09 ± 1.03). Different letters indicate significant differences ($P<0.05$).

enters and leaves the cells depends on the relative surface area available. The smaller the surface area, the lower the hemoglobin it will carry, but the gas will diffuse more easily and faster than in the larger surface area of erythrocytes. This morphometrical comparison in different species of reptiles can be explained by each species' environmental adaptation, which causes differences in physiological condition in the respiratory and gas exchange system.

Among turtles, *C. galbinifrons* and *C. porteri* are terrestrial tortoise which inhabit forests, whereas *C. mydas* is an aquatic reptile that lives in subtropical and temperate regions, coastal areas (NOAA, 2025; Arteaga & Guayasamin, 2020). According to Marn & Kooijman (2022), sea turtles and tortoises have the lowest mass-specific metabolic rates among reptiles, which allows for a longer life span. Tortoises have low energy and oxygen demand, which results from their passive physical activity and their terrestrial habitat that contains high oxygen levels. Tortoises have larger erythrocytes (*C. porteri* 114.47 ± 10.83 μm^2; *C. galbinifrons* 155.23 ± 8.12 μm^2) than *C. mydas* (84.04±7.24^{a} μm^2), which allows them to carry more gas in the cell even though they have slower oxygen diffusion. Although both sea turtles and tortoises are large reptiles (heavy body mass) and have slow metabolisms, due to sea turtles' specialized breathing and active physical activity, they have a small erythrocyte surface area, which allows more rapid oxygen diffusion.

Chelonia mydas spends most of its life cycle in the sea, diving to various depths, with some individual records reaching up to 135 m (Rice & Balazs, 2008). Oxygen diffuses more slowly through water than air, which creates a hypoxic (low oxygen level) and hypercarbic (high carbon dioxide level) environment, which causes the respiratory and gas exchange in aquatic animals to be more adapted to the habitat. *Chelonia* *mydas* have breathing episodes (intermittent breathing pattern) containing several ventilations separated by longer breath-holding periods as the physiological adaptation to the aquatic lifestyle (Trevizan-Baú *et al.,* 2018; Burggren & Shelton, 1979). During the breath-holding periods, the oxygen demand is far smaller, and it can also perform anaerobic metabolism when necessary (metabolic depression). The short period spent above the water surface and prolonged breath-holding period make the gas exchange in Chelonians' blood more rapid than that of terrestrial animals (Trevizan-Baú *et al.*, 2018). *Chelonia mydas* has the smallest erythrocyte surface area, which carries less oxygen and allows it to move across blood vessels easily and blood exchange rapidly with faster gas diffusion.

Interestingly, even though *B. constrictor* snakes also have a low metabolic rate as the other reptile species, it has the largest surface area (270.59 ± 10.12 μm^2) compared to other snakes (*X. vermicularis* and *P. najadum*) which have relatively similar surface area (**Table 2**) Variation in natural behavior of the reptiles such as hunting, eating or digesting food, and reproduction is also one of the factors affecting metabolic rate. *Boa constrictor* snakes do not have intensive physical activities as they are passive predators that use high-intensity muscle work during constriction, different from the other two snakes, which are active foragers that move continuously at lower intensity (Dupoué *et al.*, 2017; Stacy *et al.,* 2011; Arikan *et al.,* 2009). This causes boa snakes to require rapid energy turnover and large aerobic capacity compared to the other two snakes. Snakes' oxygen demand can rapidly increase up to 3 to 10 times baseline during activities and digestion, which is compromised through their cardiovascular and respiratory adjustments (Jackson *et al.,* 2015). During the fluctuation of snakes' metabolic rate, especially during digestion, they will have an increasing heart rate and oxygen demand as the body temperature increases (Last *et al.*, 2024). *Boa constrictor* often bask in the sun, which allows them to maintain higher body temperatures and raises their basal metabolic rate, while the other two snakes (*X. vermicularis* and *P. najadum*) typically live in lower temperatures and thus operate at cooler body temperatures. Based on these characteristics, *X. vermicularis* and *P. najadum* snakes' smaller erythrocyte surface area facilitates more rapid cell movement in the blood flow, which compensates for their low hemoglobin content. The low but rather fluctuative basal metabolic rate in the boa snake results in a larger erythrocyte surface area, which facilitates higher hemoglobin content so it can carry and fulfill the high oxygen demand during activity, even with a low blood circulation rate.

Unlike snakes, iguana and lizards have more active and intensive physical activities or foraging behavior, which requires higher energy and have a higher basal metabolic rate than snakes. Usually, the body temperature also increases during high metabolic rate (Mell *et al.*, 2016; Haussmann *et al.*, 2025), which requires an effective circulatory rate. This explains why lizards and iguanas have elongated erythrocytes, which help the movement of the blood cells through capillaries despite their big size more effectively. An elongated shape could help erythrocytes navigate and pass through narrow capillaries more efficiently, which reduces their resistance to blood flow and lowers the metabolic expenditure of circulation (Bury *et al.*, 2023).

Iguana iguana is an arboreal lizard that spends most of its time in trees but is mostly sedentary and has low foraging activity (Stacy *et al.,* 2011). Meanwhile, *V. niloticus* is a highly active predator that inhabits riverbanks, which support its aquatic-terrestrial habitat (Soliman *et al.,* 2023). *Varanus niloticus*, which has a more active lifestyle, supports a higher basal metabolic rate compared to the arboreal, slower-moving *I. iguana*. These differences in basal metabolic demand are shown in its erythrocyte morphometry, where *I. iguana* has a larger surface area (193.78 ± 19.22 μm^2) than *V. niloticus* (108.48 ± 15.85 μm^2) (**Table 2**).

The limitation of this study is the methodological variety in producing blood smear samples, which could potentially influence cell boundary visualization and morphometric precision. Differences in the choices of fixation solution and its duration will affect slight changes in cell morphology and color intensity of the sample, as evaporated fixation solution causes differences in osmotic pressure that could eventually cause crenation, and it allows proteins to precipitate, which affects the cellular integrity (Ghofur *et al.,* 2022; Museyaroh *et al.,* 2025). Staining variety could also affect the cell morphology, cellular integrity, and contents caused by differences in buffer systems and pH of staining (Xu *et al.,* 2022). Another limitation is related to the uncertainty of individual-level replication in the article source. In several article sources, the number of individuals contributing to each blood smear image was not specified. Consequently, measurement and statistical analysis were performed at the cellular level, which may cause pseudo-replication at the individual or population level. Acknowledging these limitations, the author made the comparison based on relative results rather than on morphometric absolute values, which are measured at the cellular level.

Conclusion

The comparative analysis of erythrocyte morphometry of several reptiles showed variation in the blood morphometry of each species. These variations reflect the evolutionary adaptation of each species to its habitat, such as climatic and behavioral factors. Smaller erythrocytes with a high erythrocyte-to-nucleus ratio were found in the sea turtle, which reflects its behavior in diving. This study showed that erythrocyte morphometry is not only characterized by its phylogenetic variety but also influenced by ecological adaptation and metabolic demand.

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Author contribution AM: Data curation, investigation, writing-original draft; KS: Conceptualization, methodology, writing-original draft; AS: Formal analysis, investigation; HM: Conceptualization, writing-review & editing; RT: Data curation, writing-review & editing; WM: Supervision, validation; DRE: Supervision, validation; ASS: Supervision, data visualization; PA: Methodology, validation; ID: Conceptualization, validation; NRA: data curation, writing-review & editing.

Availability of data and materials The data that support the findings of this study are available from the corresponding author (KS) upon reasonable request.

Declaration of generative AI in writing process During the preparation of this work, the authors used Consensus AI. After using this service, the authors reviewed and edited the content as needed and take full responsibility for the content of the published article.

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