Variation in reproductive strategies of three populations of *Phrynocephalus helioscopus* in China

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Background. Egg size and clutch size are the key life history traits. It is possible during the breeding period to increase female reproductive output either by increasing the number of eggs if the optimal egg size (OES) is maintained, or by increasing the allocation of energy to each egg. However, the strategies adopted are often influenced by their morphology and environment.

Methods. Here we examine variation in female morphological, and reproductive traits, test for an egg size-clutch size trade-off, and the relationship between egg size and female morphology in three populations of *Phrynocephalus helioscopus*.

Results. Female body size, egg size, and clutch size were larger in the Yi Ning and Fu Yun population than the Bei Tun population (the Fu Yun and Yi Ning populations laid more, rounder eggs). Egg size was not constrained by female body size in the Bei Tun and Fu Yun populations, but egg size-clutch size trade-offs occurred in both populations. Egg size-clutch size trade-offs were not found in the Yi Ning population, but egg size was correlated with female body size, consistent with the hypothesis of morphological constraint.

Conclusions. Our study found geographical variation in body size and reproductive strategies of the lizard *Phrynocephalus helioscopus*. Egg size was correlated with morphology in the larger-bodied females of the YN population but not in the small-bodied females of the BT population, illustrating that constraints on female body size and egg size do not always occur.

Variation in Reproductive Strategies of Three Populations of *Phrynocephalus helioscopus* in China

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Abstract

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1. Background

~~Animals often exhibit variation in reproductive traits~~ as a result of differences in the quality of resources and food availability of different habitats (Roff, 2002; Cruz-Elizalde & Ramırez-Bautista, 2016). Egg size and clutch size are ~~the~~ key life history traits~~,~~ and have received more attention than other reproductive traits (Qu *et al*., 2011; Amat 2008; Lovich *et al*., 2012). When food is less available~~,~~ females may face the problem of ~~having~~ limited reproductive resources to invest in eggs~~,~~ which results in a trade-off between 1) the energy allocated to each egg (egg size), and 2) the total number of eggs (clutch size, CS). An increase in resources allocation to each egg will result in decreasing CS (Roff, 1992; Kaplan & Phillips, 2006). This negative relationship between egg size and clutch size provides evidence of reproductive trade-offs (Rowe, 1992). Variation in female reproductive output is widespread, both interspecifically and intraspecifically. Especially for widespread species, local genetic variation, short-term phenotypic plasticity, and the complex interactions between these two, contribute to variation in reproductive output (Brown & shine, 2007).

Optimal egg size (OES) theory predicts that natural selection optimizes egg size within populations, such that when resources are ~~available~~ (not limiting~~)~~ for reproduction, CS or number of clutches may increase ~~rather than an increase in~~ egg size (Smith & Fretwell, 1974;

Brockelman, 1975). Natural selection predicts that females should optimize resources allocated to each egg~~,~~ and CS should only increase after ensuring the production of high quality offspring (Lovich *et al*., 2012). In some reptiles, CS is positively correlated with female ~~morphological traits~~, while egg size is ~~not~~, consistent with OES theory (Congdon & Gibbons, 1987). However, the relationship between egg size and clutch size is determined by ~~numerous~~ factors~~,~~ and the trade-offs between egg size and number are not always evident in natural populations (Berven, 1982; Liao & Lu, 2011; Wang *et al*., 2011).

~~In some reptiles,~~ egg size is correlated with female body size (morphological constraint hypothesis), and both egg size and CS increase with an increase in female body size, contrary to OES theory (Dunham & Miles, 1985; Clark, Ewert & Nelson, 2001; Mohamed *et al*., 2012; Ryan & Lindeman, 2007). When resources are limited, reproductive output is directly correlated with the trade-offs between egg size and clutch size, and ultimately with ~~the~~ offspring survival (Brown & Shine, 2009; Congdon & Tinkle, 1982). The size of each egg normally determines the success of incubation and offspring survival (Angilletta *et al*., 2004; Räsänen, Laurila & Merilä, 2005). ~~Females may allocate more energy to individual eggs, aiming for greater survival of their offspring.~~

Phrynocephalus helioscopus is a small (~~mean~~ SVL 47.5~~5~~mm) lizard that is widely distributed in Eurasia. Previous research on this species has focused on egg incubation (Wang *et al*., 2013) and female reproductive output (Liang *et al*., 2015). However, ~~among the distinct populations of this widely distributed species, neither~~ variation in ~~the~~ female reproductive traits and ~~the~~ egg size-number trade-off, ~~nor the~~ effects of female ~~morphological traits~~ on egg size have been studied. In this study, we compared female ~~morphological traits~~ and the relationships ~~among their~~ egg length (EL), egg width (EW), egg mass (EM), egg shape (ES) and clutch size (CS) in three populations. Specifically, we:

1. Tested whether reproductive female size differs among the three populations,

2. Examined how that variation is associated with reproductive traits, especially in fecundity, egg and clutch size, egg shape, and the egg size-clutch size trade-off;

3. Examined the relationship of female traits to egg and clutch size in and among populations.

2 Materials and Methods

2.1 Ethics approval

Specimens were collected following Guidelines for *Use of Live Amphibians and Reptiles in Field Research* (the Herpetological Animal Care and Use Committee (HACC) of the American Society of Ichthyologists and Herpetologists, 2004). This work was performed in compliance with ~~the~~ current laws on animal welfare and research in China. After the research was completed, ~~the~~ lizards were released where they were captured.

2.2 Study site

The populations studied here are in three ecologically distinct locations: Bei Tun city (BT: 87°15'' E, 47°26' N), Fu Yun city (FY: 89°05' E, 46°36' N), and Yi Ning city (YN: 80°47' E, 43°40' N) of the Xinjiang Uyghur Autonomous Region, China. The distance between the BT and the YN populations is about 660 km and their habitats are different. The BT population is in a typical gravel desert with little vegetation, while the YN population is in a loam desert with abundant vegetation. YN is hotter and wetter than BT. FY and BT are separated by about 160 km and FY and FY and YN by about 700 km. FY is similar to YN in vegetation and rainfall, while FY and BT have similar temperature regimes (Fig.1 and Fig.2).

2.3 Animal and egg collection

From May 2014 to May 2017, we collected *P. helioscopus* by hand from the outskirts of BT (in 2014, Liang *et al*., 2015), FY(in 2017), and YN (in 2017) and took them to Xinjiang Agricultural University, where female lizards were palpated to determine their reproductive state (Li *et al*., 2006). We collected lizards from 12:00 to 18:00~~, because both sub adlut and adult lizards during this time are active~~ (personal observation), ~~which can make sure we can capture them at random~~. Fifty-three gravid females (BT: 13, FY: 24, YN: 16) were housed individually in plastic cages in a room with ambient temperatures never above 28℃ and not lower than 20 ℃ with a 12-hour light /12-hour dark cycle. A 250 W light bulb was suspended at one end of each cage, 20 cm above the cage floor and lizards could freely move to warmer and cooler places within the cage. Mealworms (larvae of *Tenebrio molitor*) and water enriched with vitamins and minerals were provided *ad libitum*. Female ~~in cages~~ dig before they lay eggs~~,~~ ~~which~~ allowed us to collect eggs quickly, and prevented eggs from absorbing water in the moist vermiculite. ~~The cages were checked every 2 hours for eggs~~. All eggs ~~are~~ used in this study were collected no more than 20 minutes after they ~~had been~~ laid.

2.4 Morphology and Reproductive Traits

We measured female snout-vent length (SVL), tail base width (TBW), egg length (EL), and egg width (EW) by using digital calipers (measured to the nearest 0.01 mm). We also noted clutch size (CS). We weighed eggs (EM) and clutches (CM) on an electronic balance to the nearest 0.01g. The ratio of egg length to egg width (EL / EW) indicates the general shape of the eggs (egg shape, ES), where 1 is a round egg, and larger values are increasingly elongate (Ji & Wang, 2005; Kratochvíl & Frynta, 2006).

2.5 Statistical analyses

We used Levene's tests to test for heterogeneity of variances. The residuals were tested by Kolmogorov-Smirnov tests for detecting normality. We log transformed the variances to minimize the heterogeneity if necessary (King, 2000). ANOVA was used to examine differences in SVL, EM, and ES and ANCOVA was used to examine differences in TBW, EL, EW, RCM, and CS among the three populations by *post hoc* Tukey's tests (multiple comparisons). To test egg size-clutch size trade-off and analyze potential morphological constraint on optimal egg size, the relationships of EM and EL with CS, of EM with EL, of EL and CS with SVL, and of EW with TBW were examined using RMA (Reduced Major Axis regression) ~~regression~~ rather than OLS (Ordinary least squares) regression, because RMA accounts for ~~an~~ error in the independent variable (Dunham & Miles, 1985). Historical climatic data (1990-2013) of the three study areas were taken from the Chinese National Climatic Data Center (http://data.cma.cn). Descriptive statistics were represented as follows: mean adjusted (calculate by the *effect* function of "effects" package, Fox & Hong 2009) ± SE, except in SVL, EM, and ES, which are represented as the mean ± SE. Differences were considered significant when $P < 0.05$.

All analyses were conducted using R v.3.4.1 (R Core Team 2017), employing the packages "lmodel2" (Legendre, 2011), "ggplot2" (Wickham 2015), "gplots" (Warnes *et al.*, 2011).

3 Results

3.1 Female morphological variation

SVL varied between populations and was longest in the similar YN and FN populations (YN: 51.23 mm; FY: 50.43 mm), and shortest in the BT population ($F_{2,52} = 20.75$, ~~R~~$^2 = 0.45$, $P < 0.0001$, Fig. 3A). ~~ANCOVA with SVL as a covariate revealed that~~ TBW varied between populations and was smallest in the similar YN and FN populations (YN: 7.20 mm; BT: 6.93 mm), and largest in the FY population ($F_{2,52} = 6.82$, $P = 0.002$, Fig. 3B).

3.2 Female Reproductive Traits

Females in the FY population laid heavier eggs than those in the BT and YN populations. Eggs were similar in length in all three populations. Eggs were wider (rounder, EW) in FY and narrower in YN population. BT females laid smaller clutches than FY and YN females when controlling for SVL (Table1).

3.3 Egg size-clutch size trade-offs

We found a positive relationship in all populations between EL and EM (Fig. 4C). In BT and FY ~~females~~, egg size decrease with clutch size, while in YN females, egg size was independent of clutch size (Fig. 4 A, B).

3.4 The Relationship Between Egg Size, Number and Female Morphology

In the BT and YN populations, female ~~morphological traits~~ were independent of EL, EW, and CS (Fig. 5). In the YN population, while CS was independent of female ~~measurements~~ (Fig. 5 C)~~.~~ EL was weakly correlated with SVL (Fig. 5A), and EW and TBW were correlated (Fig.

5B).

4 DISCUSSION

~~We found variation in female morphological traits~~ (SVL and TBW), reproductive traits (EM, CS, RCM, and egg size), ~~in the relationship between reproductive characteristics and female morphological traits, and in~~ egg size-number trade-offs among the populations of *P. helioscopus*. Female body size, egg size, and clutch size was smaller in the BT population than the FY and YN population, and the FY and YN populations laid more, rounder eggs. Egg size was not correlated with female body size in the BT and FY populations, but egg size-clutch size trade-offs occurred in both populations. Egg size-clutch number trade-offs were not found in the YN population, but egg size was correlated with female body size.

Morphological traits, such as body size and body shape always vary among different populations in animals (e.g. Snakes: Zhong *et al*., 2017; Lizards: Horváthová *et al*., 2013; Turtles: Werner *et al*., 2016). Environmental factors that exert strong effects on animal life history traits include activity season length and food availability (Yom-Tov *et al*., 2006; Horváthová *et al*., 2013). Our study revealed that the FY and YN populations ~~have significantly larger SVLs ($P <$ 0.01)~~. Longer activity seasons were assumed to be the cause of variation in the body size between the *P. helioscopus* of the YN and BT populations (lizards in the YN population have larger SVL, Liang & Shi, 2017). Temperature, fundamentally important for lizards (Grant & Dunham 1990), was ~~higher~~ in YN ~~than the other two sites~~, especially in March and November (Fig. 2-A). In YN ~~city~~, *P. helioscopus* activity began in mid-March and hibernation began in early November, which means that the activity period for lizards here is almost a month longer than in the other two sites. Lizards in this population ~~have a larger body size might~~ because of the longer growing season ~~than the two populations~~. ~~However, differences in lizards' age among three populations were lacked in this study~~.

The BT and the FY populations with similar temperature raises the question as to what causes ~~their difference in SVL~~. One ~~plausible~~ ~~explanation~~ is that food limitation might have resulted reduced growth rates in the BT population. Rainfall is critical ~~to~~ habitat quality (e.g. vegetation cover and prey abundance, Lorenzon, Clobert & Massot, 2001).~~ G~~eographic variation in rainfall ~~in our study areas~~ is great (Fig. 2) and sparse vegetation in BT is due to drier conditions versus the more abundant vegetation in the FY and YN sites (Fig. 1, Fig. 2-B). Humidity is the most important factor influencing abundance and distribution of insects (Savopoulou-soultani *et al.*, 2012; Cesne, Wilson & Soulier-Perkins, 2015) and so drier conditions and sparse vegetation should be associated with less food.

Egg size varies among populations because of variation in female body size and is ~~considered to be~~ an important female trait that can affect offspring ~~size~~ (Morrison & Hero, 2003; Olsen & Vollested, 2003; Steyermark & Spotila, 2001). ~~Our data showed~~ that egg size differed among the three populations, which suggest that larger females of the FY and YN populations are able to ~~devote~~ more resources to eggs production. In addition, egg size is also correlated with the incubation period, with smaller eggs having a relatively short incubation time (Thompson & Pianka, 2001). Perhaps the BT population smaller eggs hatch sooner providing offspring time to forage before entering hibernation. EL ~~did not significantly differ~~ among the three populations (EM as the covariate), but EW ~~did~~. On the other hand, egg shape is also related to clutch size and larger clutches tend to have more rounded eggs (Ji *et al*., 2002). So, eggs were narrower in BT, and both the FY and YN populations lay more, rounder eggs (Table 1).

Larger females tend to lay more eggs in reptiles (Amat, 2008; Ryan & Lindeman, 2007). Thus, the smaller CS of the BT population ~~is~~ associated with their body size~~. CS can also be limited by food availability, and varies among populations~~ (Liao, Lu & Jehle, 2014; Roitberg *et al*., 2015). ~~The BT population can have smaller females and clutches due to shorter growing seasons and limited food availability~~.

The trade-off between egg size and clutch size is an important concept in life-history theory (Kern *et al*., 2015). Egg size and clutch size were negatively correlated in the BT and FY populations (EM and EL), but not in the YN population. In the YN population there was no egg size-clutch size trade-off, and so ~~intraspecific variation in the relationship between egg size and clutch size is widespread~~ (Liao, Lu & Jehle, 2014; Roitberg *et al*., 2015).

Generally speaking, offspring phenotypes are influenced by female body size (e.g., SVL, Krist & Remeš, 2004). ~~Morphological traits~~ and other factors affecting egg size will result in the following five possible outcomes (Lovich *et al*. , 2012): 1) egg size is constrained by female morphology (not optimized), 2) egg size is unconstrained by female morphology (optimized), 3) egg size is unconstrained by female ~~morphology~~ and optimized only in the largest females (Fehrenbach *et al*., 2016), 4) egg size is not constrained by the pelvic aperture width, and is not optimized, but rather is constrained by some other non-morphological factor (e.g., age or clutch number, Clark, Ewert & Nelson, 2001; Paitz *et al*., 2007; Harms *et al*., 2005), 5) egg width is constrained and requires osteo-kinesis for oviposition (Hofmeyr, Henen & Loehr, 2005;

Fehrenbach *et al*., 2016).

Consistent with the prediction of the morphological constraint hypothesis, egg size increases as the size of the female increases (~~outcome~~ 1) in the YN population. Although female body size in the BT population is smaller than in the FY population, in both cases, their egg size was uncorrelated with female body size (outcome 2 or 4 above). For some species with small body sizes, egg size is constrained by female morphology (Ryan & Lindeman, 2007). In small-bodied females, the body size-specific constraints on egg size coupled with selection towards an optimum egg size results in a positive correlation between body size and egg size. Egg size (EL and EW) was not dependent on female body size in either the BT or FY population, but there were ~~significant~~ negative correlations between egg size and number (Fig. 4), suggesting that ~~the~~ egg size was constrained by CS (non-morphological factor) in both populations (Brown & Shine, 2009, outcome 4). (Smith & Fretwell 1974; Congdon & Gibbons, 1987). Unexpectedly, ~~our results revealed~~ that egg size was correlated with the large-bodied females of the YN population. A positive relationship between egg size and female size indicates that there is no optimal egg size, as in the YN population (Escalona, Adams & Valenzuela, 2018). But we found some support for the prediction that EW was constrained by TBW (Fig. 3), since eggs must fit the female tail base width which they pass through on their smallest axis (e.g. EW). In some turtle species, EW but not EL increases with the size of the female (Rasmussen & Litzgus, 2010). There was a significant positive (but weak) correlation between EL and female SVL in the YN population, suggesting that EL is dependent upon on female SVL. EL can be constrained by morphological factors, non-morphological factors (e.g. CS), or their interactions, which may indicate that a weak relationship between female morphology and EL in the YN population. The specific mechanisms of the non-morphological factors require further study (Kern *et al*., 2015).

CONCLUSIONS

In summary, we found geographic variation in body size and reproductive strategies of the lizard *Phrynocephalus helioscopus*. Lizards in populations with longer growing seasons and abudant vegetation (the FY and YN populations) are larger. Lizards of the BT population are smaller (perhaps due to food limitation or season limitation) also have smaller clutches than the FY and YN populations. FY and YN produce rounder eggs, perhaps due to larger body size. This study found that egg size was correlated with female body size in the larger-bodied females of the YN population – an anomaly for the morphological constraint hypothesis. Egg size was not correlated with female body size and did not follow the optimal egg size hypothesis in the BT and FY populations. Egg size-clutch size trade-off suggests that egg size was constrained by CS in both populations.

However, whether the existence of genetic variation is related to the differences in the life history traits of the three populations of this species has not been examined in this study and should be researched in the future.

ACKNOWLEDGEMENTS

We are grateful to Prof. Lovich of the United States Geological Survey, to the Southwest Biological Science Center, and to anonymous reviewers for their excellent help in improving this manuscript. We thank Luo D and Wang P for assistance during fieldwork, and we thank An J for help with the egg collection and lizard husbandry. Mr. T. Martin provided professional advice regarding spelling and phrasing.

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Figure 1

Map, showing the three locations where lizards were captured for this study in the Xinjiang Uyghur Autonomous Region of western China.

Closest Cities (BT, FY, and YN) are identified by the red dots, and the collecting locations are indicated by the black dots with arrows. Photos indicate habitat types in each sampling location (Photo credit: Tao Liang).

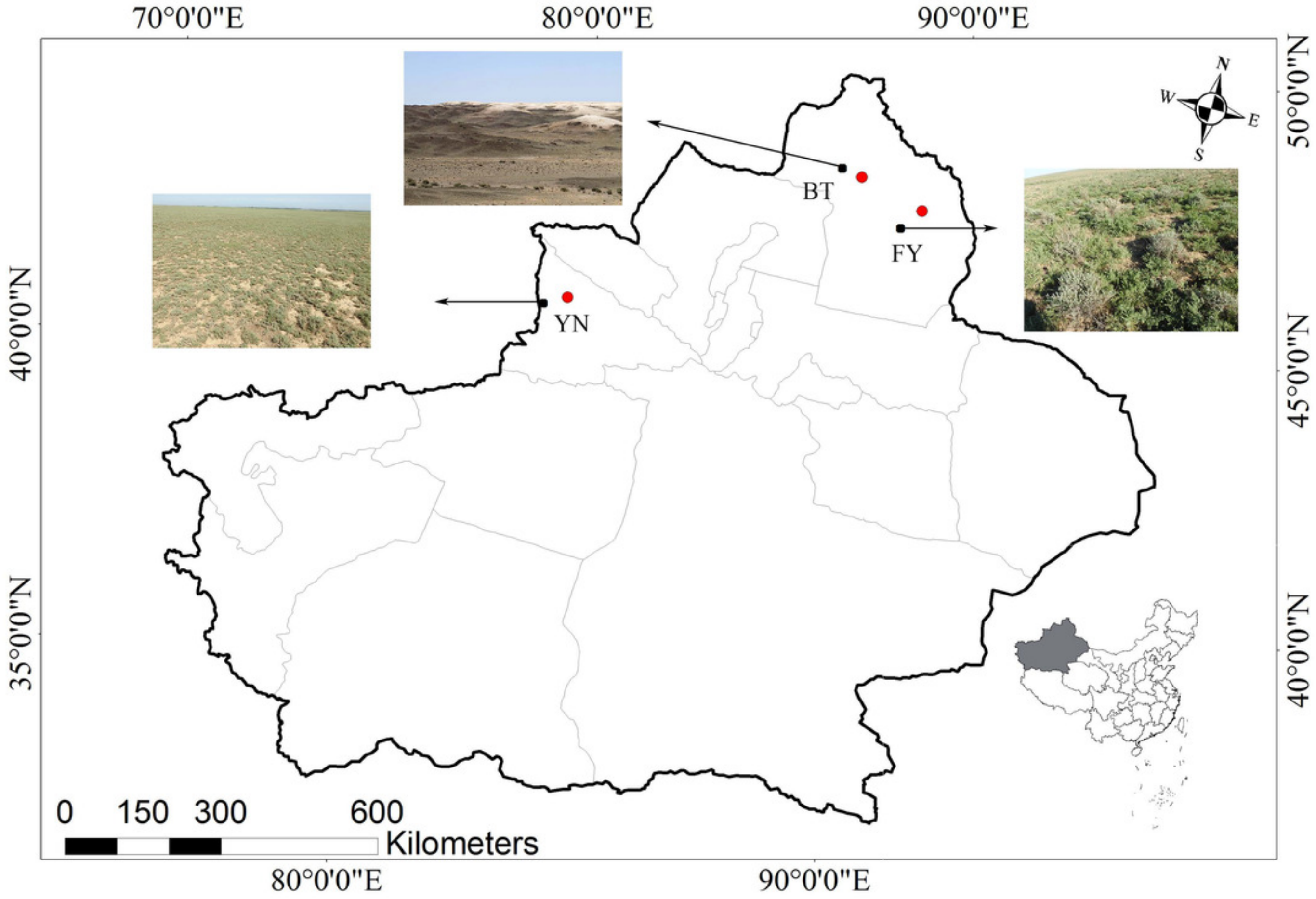

Figure 2

Means for monthly mean air temperature (A) and monthly mean rainfall (B) over the past 24 years (1990-2013) at the three localities~~, where females of *Phrynocephalus helioscopus* were collected~~.

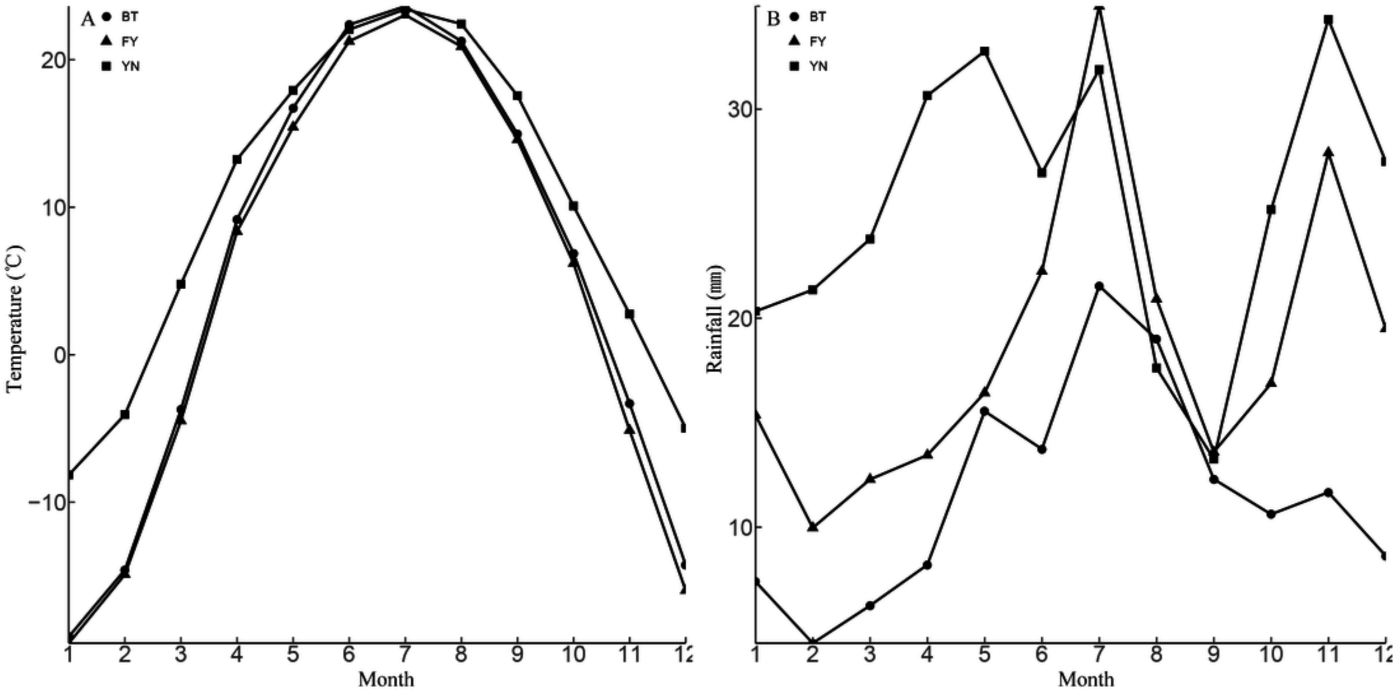

Figure 3

Comparisons between A) snout-vent length and B) tail width at base, of gravid females in three populations of *Phrynocephalus helioscopus*. Points are means with 95% confidence intervals. Different letters means significant at the 0.05 level.

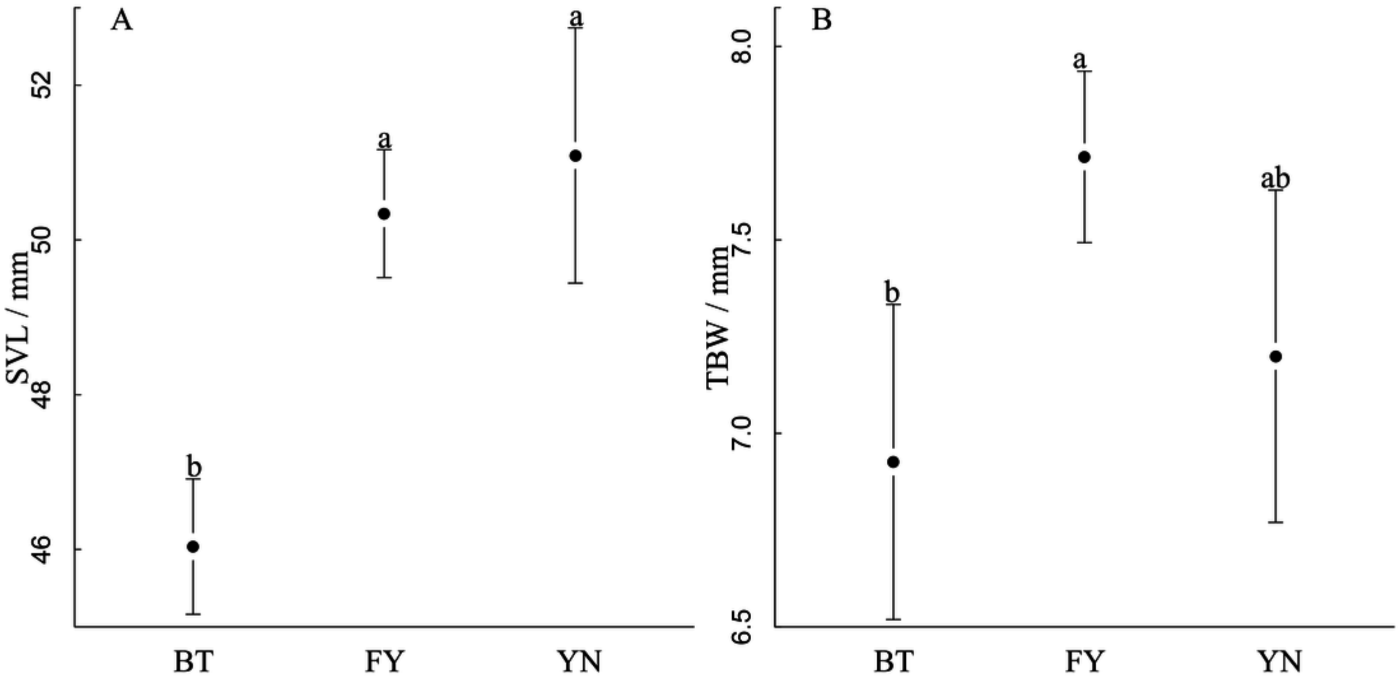

Figure 4

Regressions of EL and EM and egg size-number trade-off of *Phrynocephalus helioscopus*.

Fitted reduced major axis regression model (when $P < 0.05$) and statistical significance are indicated in each case. BT - Shaded circle, FY - Shaded triangle, YN – Shaded square.

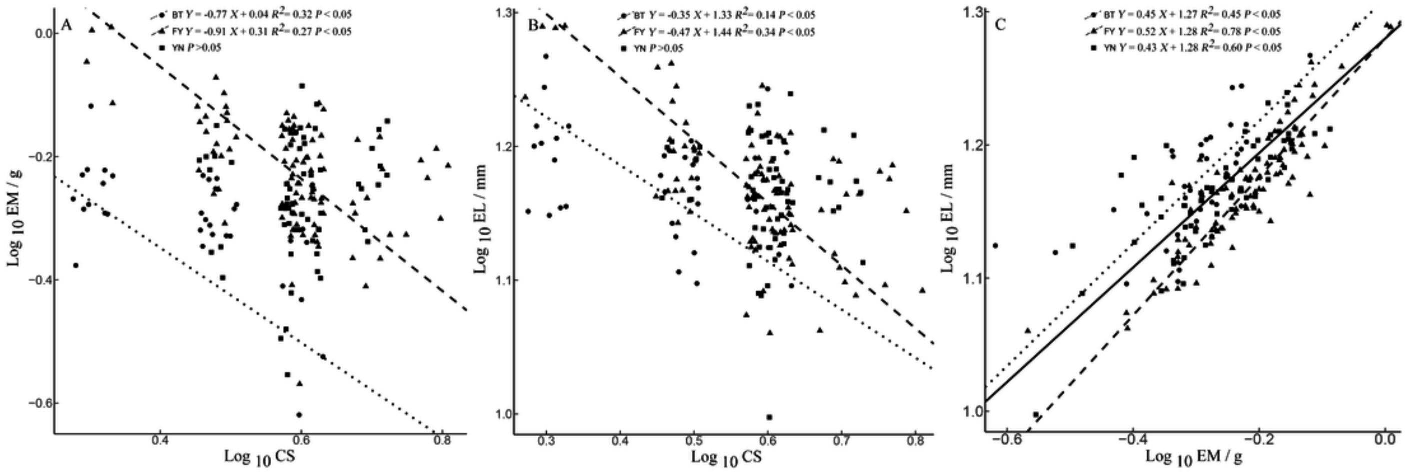

Figure 5

Regressions of egg length (A), egg width (B), and clutch size (C) and female morphological traits from three populations of *Phrynocephalus helioscopus*.

Fitted reduced major axis regression model (when $P < 0.05$) and statistical significance are indicated in each case. BT - Shaded circle, FY - Shaded triangle, YN – Shaded square.

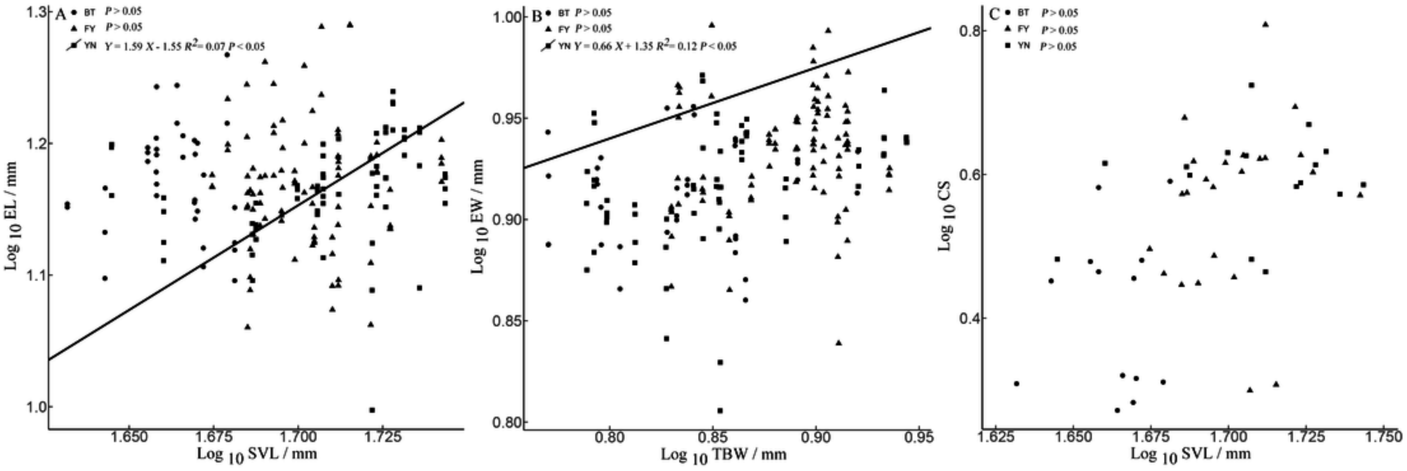

Table 1(on next page)

Descriptive statistics of female reproductive traits in the three populations of *Phrynocephalus helioscopus*

Different letters means significant at the 0.05 level;

† ANOVA;

One-way analyses of covariance (ANCOVAs) (for CS with SVL as the covariate, for EL and EW with egg mass as the covariate);

* BT n=13, FY n=24, YN n=16.

	BT (n=35)	FY (n=90)	YN (n=63)	*F*-level and *P*-value
EM (g)†	0.51±0.02^{b}	0.61±0.02^{a}	0.55±0.01^{b}	$F_{2,187}$=11.67, R^2 = 0.11, $P < 0.0001$
range	0.32～0.76	0.27～1.02	0.28～0.82	
EL (mm)#	15.66±0.24^{a}	14.39±0.17^{a}	14.91±0.16^{a}	$F_{2,187}$ = 1.15, P = 0.318
range	12.47～18.51	11.49～19.50	9.94～17.35	
EW (mm)#	8.41±0.08^{b}	8.45±0.06^{a}	8.34±0.07^{b}	$F_{2,187}$ = 19.42, $P < 0.0001$
range	7.19～9.03	6.90～9.90	6.39～9.36	
ES†	1.83±0.03^{a}	1.73±0.02^{b}	1.78±0.02^{ab}	$F_{2,187}$=6.71, R^2 = 0.06, $P < 0.0001$
Range	1.44~2.27	1.43~2.18	1.47~2.11	
CS#*	2.93±0.13^{b}	3.69±0.18^{a}	3.82±0.14^{a}	$F_{2,187}$ = 10.93, P = 0.0001
range	2～4	2～6	3～5	