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Research Article
Relationships among Sperm-Egg Penetration, Fertility and Egg Components of Chinese Painted Quail (*Coturnix chinensis*)[1]

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Abstract

Background and Objectives: Birds are known to exhibit physiological polyspermy and store sperm in the female reproductive tract. The Chinese painted quail (*Coturnix chinensis*) serves as an excellent experimental animal model for avian reproduction studies due to its small size and rapid sexual maturity. However, information regarding the duration of fertility and the minimum number of sperm-egg penetration (SEP) holes for maximum fertility is not available for this species. Also, it is unknown if components of freshly laid or incubated eggs are altered due to the intensity of SEP and subsequent embryonic development. Therefore, the objectives of this study were to determine the duration of fertility following male removal, the minimum SEP holes required for maximum fertility and the relationship of egg components with SEP and fertility in Chinese painted quail. **Materials and Methods:** From 60 breeding pairs, eggs were collected daily, labeled and weighed. Weights of albumen, yolk and shell were obtained, in addition to albumen pH, height and percentage solids as well as shell thickness. On alternate days, eggs were analyzed for SEP and fertility. To obtain duration of fertility, males were separated from the hens after obtaining at least 3 eggs for SEP per breeding pair and analysis continued until SEP was 0 for 3 consecutive eggs. **Results:** We observed that the duration of fertility was 9 days and the minimum SEP for >95% fertility was ~75 holes. Further, we report, for the first time, correlations between various egg components and intensity of SEP, fertility and duration of fertility. Additionally, various egg components were found to be correlated with intensity of SEP and fertility. **Conclusion:** It appears that sustained sperm storage is very inefficient in Chinese painted quail when compared to its close relative, the Japanese quail and other avian species in general. Also, it appears that embryonic development affects egg component weights and characteristics possibly by altering transit time of the egg through the oviduct.

Key words: Chinese painted quail, egg components, embryo, fertility, sperm storage, sperm-egg penetration

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Data Availability: All relevant data are within the paper and its supporting information files.

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INTRODUCTION

Chinese painted quail *(Coturnix chinensis)* are found from India to southeast China and down into Australia. Recently, they have served as a superb experimental animal model for avian reproduction studies due, in part, to their short incubation interval, rapid sexual maturity and very small size[1]. They are the smallest of all species in the order Galliformes and the adults weigh ~50 g[1]. Moreover, they have been extensively used for research on avian parthenogenesis[2,3] and are known to exhibit spontaneous parthenogenesis, embryonic development in unfertilized eggs without any contribution from the male[2,4]. Even though multiple studies on parthenogenesis have been conducted in this species, much information on fertilization and other reproductive mechanisms of this species are still unknown[5].

In avian species, for a successful fertilization, spermatozoa must fuse with the female pronucleus within 15-30 min of ovulation[6]. In part because the fertilization window is very narrow, avian species have developed two physiological mechanisms to enhance their chances of successful fertilization: sperm storage in the female reproductive tract[7,8] and polyspermy [9,10].

For sperm storage, female birds possess sperm-storage tubules (STTs) at the utero-vaginal junction and sperm nest at the distal infundibulum, the site of fertilization[7,9,8]. The SSTs are the primary sperm storage structures and allow sustained fertility in avian species even in the absence of males[11]. However, the duration of sperm storage in the SSTs and thereby the duration of fertility after a single mating or artificial insemination varies with species. In fact, the duration of fertility is strongly correlated with the number of SSTs, which in turn is correlated with body size[12,13]. Further, the duration of fertility is up to 10-13 days in finches[14] and Japanese quail[15], 3-4 weeks in chickens[16] and 8-15 weeks in turkeys[17]. Hence, for both domesticated and non-domesticated birds, the duration of sperm storage in the female reproductive tract has been used to develop practical strategies of reproduction[18,19].

In physiological polyspermy, multiple spermatozoa digest and penetrate the inner perivitelline layer around the germinal disc area immediately after ovulation[20]. However, the male pronucleus of only one spermatozoon fuses with the female pronucleus of the ovum[20,21] and the remaining excess spermatozoa undergo degradation by DNase activity[22]. Immediately after fertilization, the outer perivitelline layer is formed around the ovum, trapping many sperm and preventing any additional sperm entry[23]. Estimation of the number of holes caused by the penetration of spermatozoa through the inner perivitelline layer (Sperm-egg penetration, SEP) and the number of spermatozoa trapped on the outer perivitelline layer are the best indicators to determine true fertility[24]. In fact, there is a positive correlation between SEP and the number of spermatozoa trapped on the outer perivitelline layer[24]. Moreover, a minimum number of sperm must penetrate the germinal disc area to ensure maximum fertility. For instance, in chickens a minimum of 30 SEP/1.35 mm^2 germinal disc area is required to attain >95% fertility[25]. Further, in Japanese quail, the minimum number of spermatozoa trapped on the outer perivitelline layer to ensure >95% fertility was 3/mm^2 of the germinal disc[26].

In a comparative study of 27 species of birds, including both passerine and non-passerine birds, Birkhead *et al.*[24] reported a positive correlation between SEP, ovum size and body mass. Additionally, a recent study conducted by Hemmings and Birkhead[27] reported that the intensity of SEP of zebra finches and domestic fowl is related to embryo survival during the early stages. However, it is unknown if any other components of freshly laid or incubated eggs, such as albumen and shell, are altered due to the intensity of SEP and subsequent embryonic development. Moreover, the absence of sperm-egg interactions occasionally leads to avian parthenogenesis which is usually abortive in nature[4,28]. However, Chinese painted quail parthenogens are known to alter albumen characteristics similar to early fertilized embryos[3,5], therefore the intensity of SEP may yield changes in other egg components as well. Additionally in mated Chinese painted quail, Santa Rosa *et al.*[5] reported a wide variation in SEP, ranging from 0 to 2000 sperm holes. However, the minimum number of spermatozoa required to ensure fertilization are unknown for this species. Therefore, the objectives of the present study were to determine (1) The minimum SEP holes required to yield maximum fertility, (2) The duration of fertility following male removal and (3) The relationship of egg components with the intensity of SEP and fertility in Chinese painted quail.

MATERIALS AND METHODS

Housing and egg collection: A random breeding population of Chinese painted quail were used in this study. From hatch until 4 week of age, both males and females were brooded together and were fed a commercial quail starter diet ad libitum. At 4 week of age, when male plumage was visible and prior to sexual maturity, females were separated from the males and placed into colony cages. At 6 week of age, females were moved to individual cages to monitor their egg production and once they started laying, females were housed

with males in individual cages (single mating pairs). Beginning at 4 week of age, both males and females were fed a commercial quail breeder diet ad libitum and were exposed to 17 h of light. A total of 60 breeding pairs were used. Daily, fresh eggs were collected, labeled and weighed. On alternate days, fresh eggs were subjected to SEP analysis and incubated eggs were subjected to fertility analysis. After obtaining 3 eggs of SEP per breeding pair, males were removed. Next, to estimate the duration of fertility following male removal, SEP and fertility analyses were continued until SEP was 0 holes (infertile) for 3 consecutive eggs[28]. All the birds used in this study were treated in accordance with the Guide for Care and Use of Laboratory Animals[29].

Egg component and sperm-egg penetration (SEP) Analysis: A total of 456 eggs from 60 breeding pairs were subjected to SEP analysis. As fresh eggs were broken open for SEP analysis, albumen pH was determined using pH strips[30] and albumen height was determined using a tripod micrometer[31]. In addition, total egg weight, yolk and shell weights as well as shell thickness were measured[32]. Albumen weight was calculated by subtracting yolk and shell weights from the total egg weight. To determine percentage albumen solids, albumen following separation was weighed before and after drying in an electric oven at 110°C for 24 h[1]. To perform SEP analysis, the perivitelline layer was processed as described by Santa Rosa *et al.*[5] and the number of sperm holes were counted in 1.35 mm^2 germinal disc area using a light microscope at 40x magnification.

Fertility analysis: To examine embryonic development, 532 eggs from 60 breeding pairs were incubated at 37.5°C and 50% relative humidity. At 10 days of incubation, eggs were candled and the eggs that were clear or showing little to no embryonic development were broken open to determine if they were fertilized. Further, because a few egg shells were so dark at candling that embryonic development could not be clearly discerned, hatch residue analysis was also performed to determine whether the eggs that failed to hatch were fertilized[30].

Statistical analysis: Data were analyzed with a completely randomized design using SAS version 9.4[33]. Population distribution of SEP before male removal was determined, for individual eggs as well as for hen averages. SEP and fertility means for each day post-male removal were determined and regression analyses were used to examine the relationships of SEP and fertility with days following male removal. Next, the duration of fertility following male removal and the minimum number of sperm required for maximum fertility were determined. Correlation analyses were used to examine the relationships of egg components with intensity of SEP and fertility.

RESULTS

Population distribution of SEP prior to male removal: Prior to male removal, the population distribution of SEP holes in individual eggs was skewed greatly to the right and ranged from 0-1900 holes with a median of 72 and a mean of 192±293 (Fig. 1a). Additionally, great variation was also seen in the population distribution of SEP averages in hens, which ranged from 0-977 holes with a median of 105 and a mean of 181±224 (Fig. 1b).

Relationship between SEP and fertility post-male removal: A quadratic decline in SEP over days post-male removal was obtained, beginning at 275 SEP holes and declining to 0 holes by 9 d (R^2 = 0.84, p = 0.001; Fig. 2a and 3). However, fertility declined linearly over days post-male removal, beginning at 100% and reaching 0% by 9 d (R^2 = 0.90, p<0.001; Fig. 2b). Hence, the duration of fertility following male removal was 9 days. Further, a positive quadratic relationship between SEP and fertility existed, such that fertility showed a linear increase

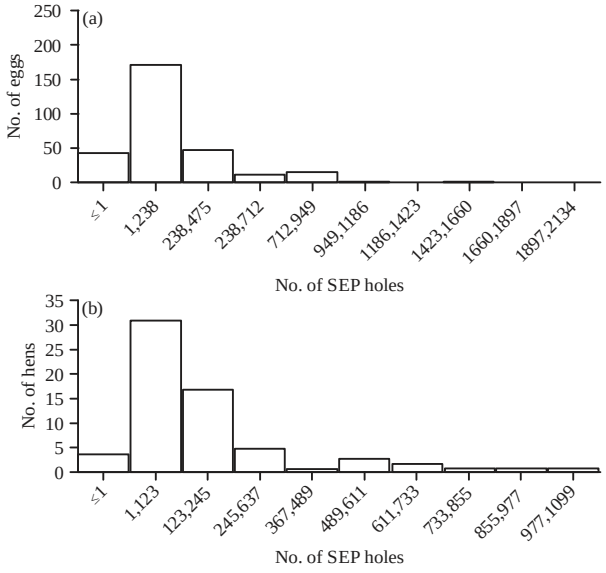

Fig. 1(a-b): Sperm-egg penetration population distribution prior to male removal

(a) Population distribution for individual eggs with sperm-egg penetration ranging from 0-1900 with a mean of 192±293 and a median of 72 sperm holes, (b) Population distribution for hen means with sperm-egg penetration ranging from 0-977 with a mean of 181±224 and a median of 105 sperm holes

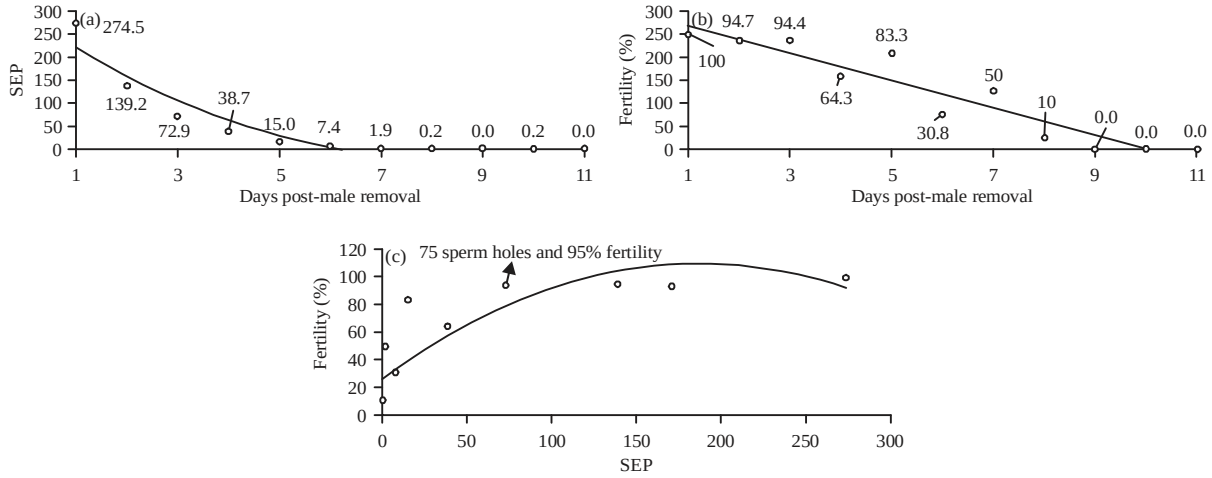

Fig. 2(a-c): Relationship between sperm-egg penetration (SEP) and fertility following removal of male

(a) A quadratic decline in SEP following male removal and approaches 0 sperm holes by 9d ($y = 2.30x^2-53.85x+228.98$, $R^2 = 0.84$, $p = 0.001$). (b) Linear decline in candling fertility following male removal and reaches 0% fertility by 9 days ($y = -10.88x+111.62$, $R^2 = 0.90$, $p<0.001$). (c) A quadratic increase in fertility as SEP increases from 0 sperm holes ($y = -0.0024x^2+0.897x+26.115$, $R^2 = 0.70$, $p = 0.01$) to 75 sperm holes and the minimum SEP required for >95% fertility is ~75 sperm holes

initially and reached a maximum as SEP increased but beyond 75 SEP holes there was no substantial increase in fertility ($R^2 = 0.70$, $p = 0.01$; Fig. 2c). Therefore, the minimum SEP for >95% fertility was ~75 holes in Chinese painted quail.

Relationship of egg components with SEP and fertility: The relationships of egg components with SEP and fertility are presented in Fig. 4 and 5. Positive linear correlations were observed for egg weight ($r = 0.13$, $p = 0.09$; Fig. 4a), yolk weight ($r = 0.16$, $p = 0.04$; Fig. 4b) and albumen height ($r = 0.20$, $p = 0.01$; Fig. 4c) with SEP. A quadratic decline was observed for percentage albumen solids with SEP ($r = 0.35$, $p = 0.04$; Fig. 4d). Further, fertility showed a positive quadratic correlation with egg, yolk, albumen and shell weights and a positive linear correlation with shell thickness (Fig. 5).

DISCUSSION

The current study showed that in Chinese painted quail the duration of fertility following male removal was 9 days and the minimum number of sperm-egg interactions required to insure maximum fertility was 75 sperm holes. It appears that Chinese painted quail have a shorter duration of fertility and require more sperm to penetrate the germinal disc area for a successful fertilization compared to other avian species in the same family, like chickens and Japanese quail[25,26]. Chickens require a minimum 30 sperm holes/1.35 mm^2 around the germinal disc in the inner perivitelline layer and Japanese quail require minimum 3 sperms/mm^2 on the outer perivitelline layer of the germinal disc to insure >95% fertility[25,26]. Moreover, in chickens and Japanese quail the number of sperm trapped in the outer perivitelline layer was correlated with the sperm holes in the inner perivitelline layer[34,35]. Previously, Bakst *et al.*[36] hypothesized that the duration of fertility is associated with the number of SSTs and that a greater number of SSTs result in greater storage space and a slower daily release of sperm. In fact, the number of SSTs in the utero-vaginal junction varies between species[36,12] and is positively correlated with body size[13]. For instance, on average, zebra finches have 1,500, broiler breeder chicken hens have 4,900 and turkeys have 30,600 SSTs[36,12]. Probably the greater body size of turkeys is responsible for the greater number of SSTs allowing for longer sperm storage and thus, longer duration of fertility compared to other smaller avian species[37]. The average body weight of adult Chinese painted quail hens is only 50g[1]. Because of their smaller body size, it is likely to have a smaller number of SSTs as opposed to chickens and turkeys, thus making sustained sperm storage very inefficient in Chinese painted quail hens. Another plausible explanation for the shorter sperm storage in Chinese painted quail could be the release of an excessive number of sperm from the SST, because they require a large number of sperm to ensure fertilization as compared to chickens and Japanese quail[25,26]. However, additional research investigating the distribution and number of SSTs and the mechanism of sperm release from SSTs are required to confirm why this species requires more sperm to ensure fertilization. In addition, in the present study, the population distribution of SEP showed a wide variation, ranging from 0-1900 for individual eggs and 0-977 for SEP

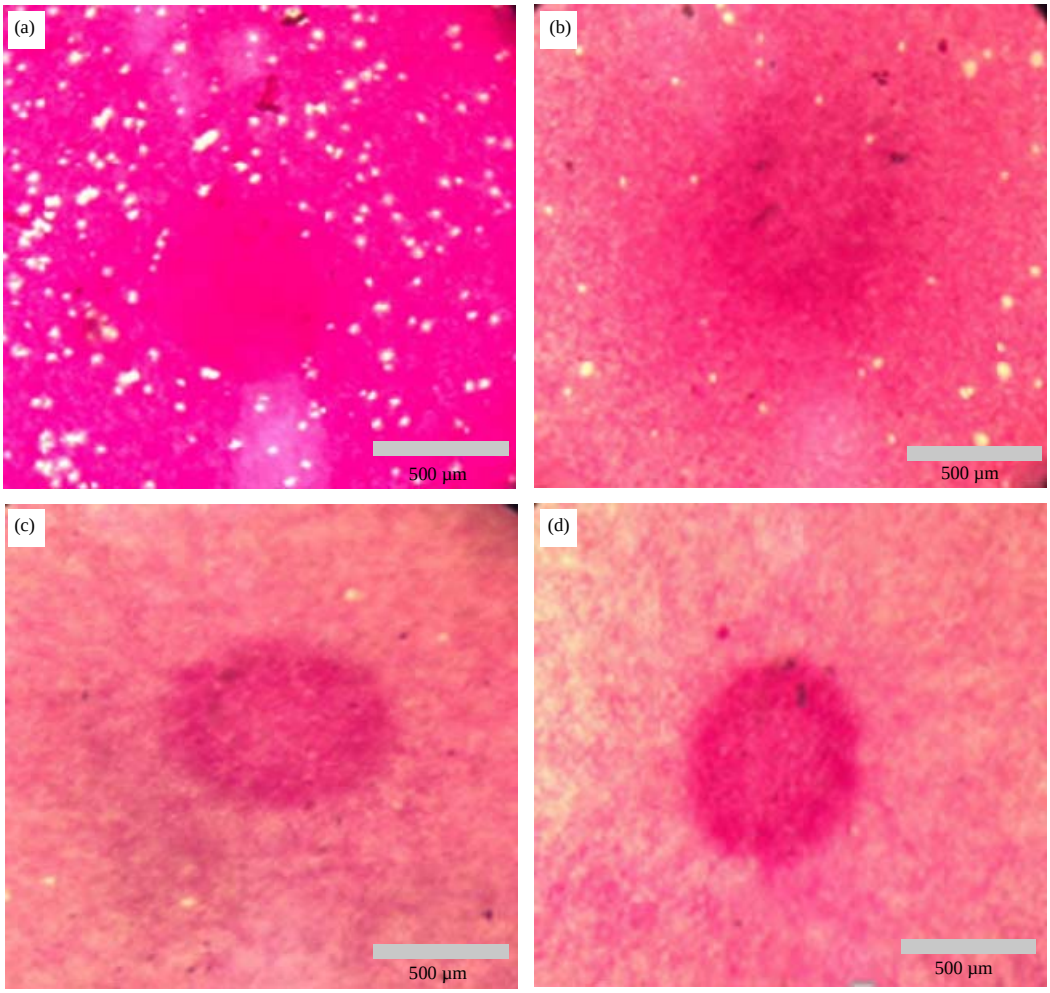

Fig. 3(a-d): Sperm penetration through the perivitelline layer surrounding the germinal disc of eggs laid on days following male removal

(a) Numerous sperm holes on the perivitelline layer on day 1 after male removal, (b) A decline in sperm holes around the germinal disc area on day 4 after male removal, (c) Very few sperm holes on the perivitelline layer on day 7 post-male removal, (d) No sperm holes around the germinal disc area on day 9 post-male removal. White dots are sperm holes where light from the microscope passes through the perivitelline layer. The dark circle is the center of the germinal disc

averages in hens. Recently, a similarly wide variation in SEP was reported by Santa Rosa *et al.*[5] in a random bred population of Chinese painted quail as well as those selected for parthenogenesis. It appears that this wide variation in SEP holes is probably due to their inefficiency for sustained sperm storage and their requirement of a large number of sperm to attain fertilization. Moreover, both virgin and mated Chinese painted quail hens are known to exhibit parthenogenesis[2,38]. Again, parthenogenesis is embryonic development that occurs without SEP or any participation of the male[4]. It is possible that in Chinese painted quail, parthenogenesis was adopted as an alternative mode of reproduction for the existence of the species, because their sustained sperm storage is very inefficient. In the absence of a male, they may deplete spermatozoa quickly and hence, there is a need for an alternative form of reproduction.

In the current study, several correlations among egg components, SEP and fertility were obtained. For example, egg weight and yolk weight were positively correlated with SEP and fertility. This result agrees with a previous study conducted by Birkhead *et al.*[34] who studied 27 different species of birds and reported a positive correlation between SEP, ovum size and body mass. It is possible that greater distention of the infundibulum caused by a larger ovum size may lead to a greater number of sperm penetrating the egg at the infundibulum and ultimately a larger egg

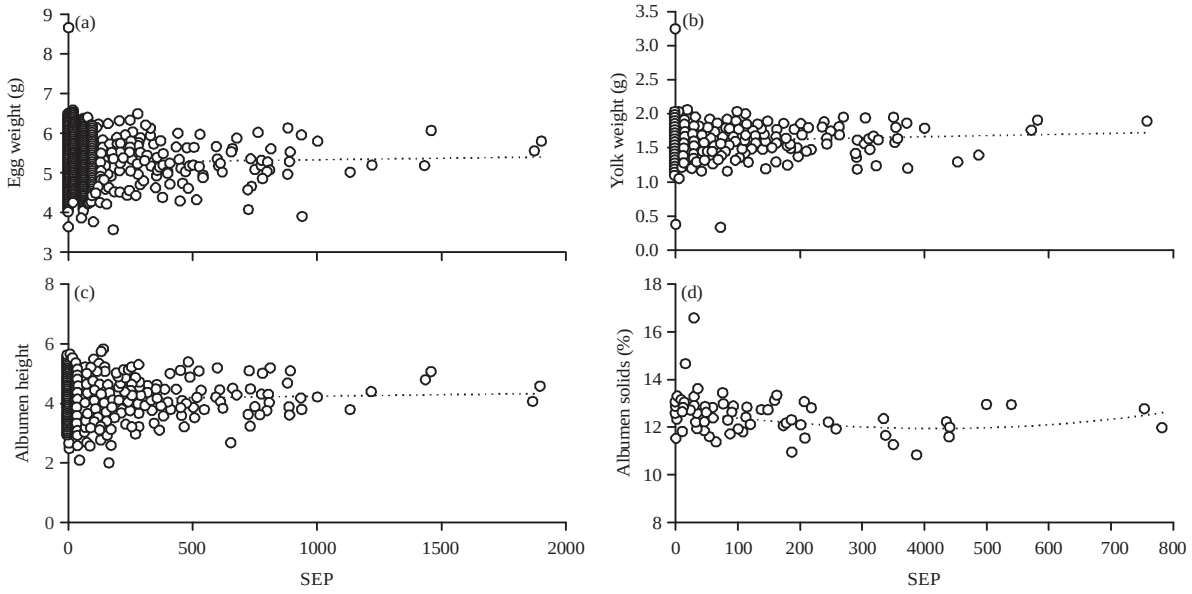

Fig. 4(a-d): Relationship of egg components with sperm-egg penetration (SEP)

(a) A positive linear relationship exist between total egg weight and SEP (y = 7E-05x + 5.26, r = 0.13, p = 0.09), (b) A linear increase in yolk weight was observed with increase in SEP (y = 8E-05x+1.60, r = 0.16, p = 0.04), (c) There is a positive linear relationship between albumen height and SEP (y = 9E-05x+4.1449, r = 0.20, p = 0.01), (d) A quadratic decline in percentage albumen solids observed with increase in SEP (y = 5E-06x^2-0.0043x+12.894, r = 0.35, p = 0.04)

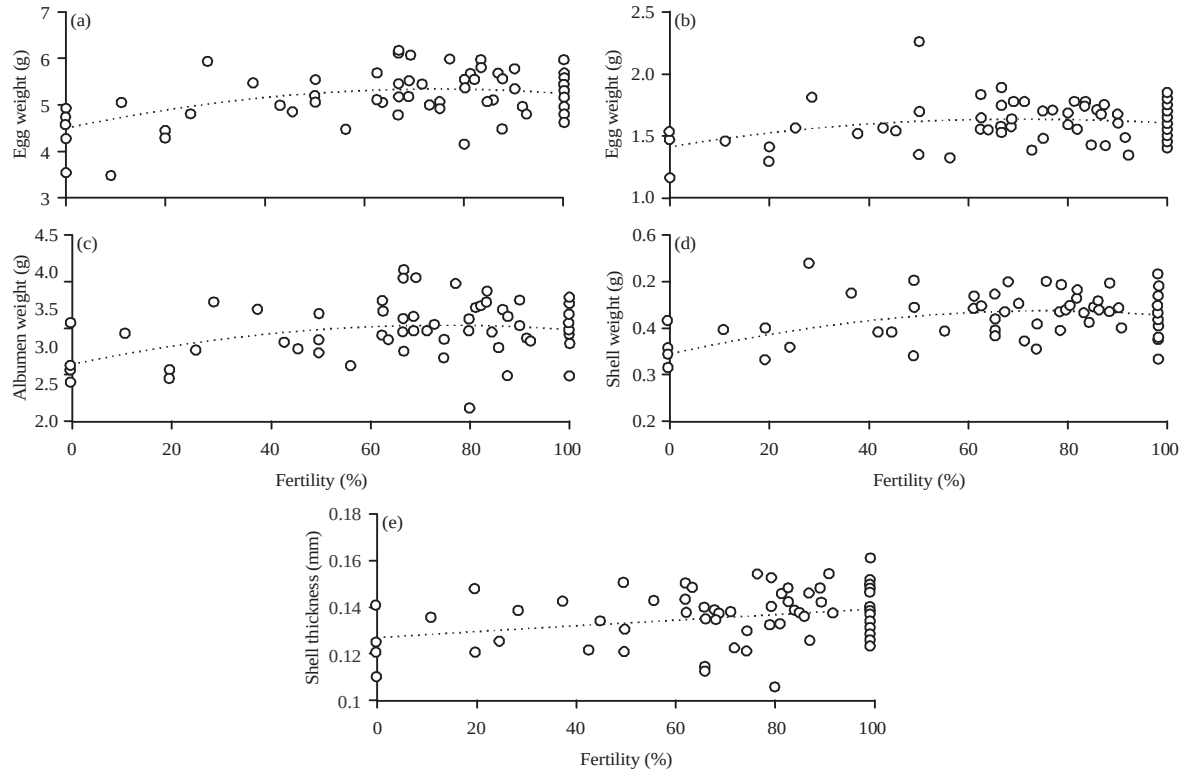

Fig. 5(a-e): Relationship of egg components with fertility

(a) There was a positive quadratic relationship for total egg weight with fertility (y = -0.0001x^2+0.022x+4.52, r = 0.47, p = 0.01), (b) A positive quadratic relationship exist between yolk weight and fertility (y = -4E-05x^2+0.0063x+1.41, r = 0.36, p = 0.05), (c) A positive quadratic relationship exist between albumen weight and fertility (y = -9E-05x^2+0.014x+2.765, r = 0.40, p = 0.05), (d) A positive quadratic relationship exist between shell weight and fertility (y = -2E-05x^2+0.0024x+0.3438, r = 0.50, p = 0.01), (e) A positive linear relationship exist between shell thickness and fertility (y = 0.0001x+0.13, r = 0.34, p = 0.005)

weight[39]. Further, there exists a positive correlation between SEP and fertility, as the number of sperm penetrating the germinal disc area increases there is a greater chance of a successful fertilization[35]. Following a successful fertilization after SEP, oviductal embryonic development may begin utilization of albumen proteins[40] and was probably responsible for the quadratic decline in percentage albumen solids (protein) with increasing SEP. Also, a greater ovum size is known to cause a greater distention of the uterus[41], thus stimulating a greater secretion of plumping fluid by the uterus[42] which might have diluted the albumen protein concentration and thus, caused a further decline in percentage albumen solids. Furthermore, the developing embryo could influence other albumen characteristics as well. For instance, there exist a positive association between SEP and albumen height as well as fertility and albumen weight. Interestingly, Santa Rosa *et al*. [5] reported that quail parthenogens, which are viable embryos, modify albumen characteristics, such as pH, O_2, CO_2 and Cl^- similar to early fertilized embryos. Further, similar to the normal fertilized embryos in the current study, quail parthenogens also utilize albumen proteins from an early stage[5]. In addition, quail parthenogens appear to alter the transit time of the egg through the oviduct, allowing a longer stay in the magnum and uterus, thus, resulting in greater albumen and shell secretions, respectively[32]. Similar to quail parthenogens, possibly normal fertilized embryos in the current study, also increased the egg transit time in the magnum resulting in a greater albumen weight. Additionally, an increased amount of plumping fluid from the uterus might have further contributed to increased albumen weight[16]. Fertility was found to have a positive association with shell weight and thickness. This is also possibly due to an altered egg transit time through the oviduct induced by the embryo, resulting in a longer stay in the uterus and, thus, yielding greater shell secretions[32].

CONCLUSION

In Chinese painted quail, the duration of fertility following male removal was found to be 9d. Further, minimum SEP holes required around the germinal disc area to attain >95% fertility was observed to be ~75 holes. It appears that Chinese painted quail have a shorter duration of fertility and require more SEP to attain maximum fertility when compared to other galliform species. It is possible that because such a large number of sperm are required to ensure fertilization that, with each ovulation, the hen may release an excessive number of sperm from the SSTs. This excessive release of sperm would make sustained sperm storage very inefficient in this species. Additional research on the number of SSTs and the mechanism of sperm release from the SSTs in this species are required to confirm our hypothesis. Further, various egg components were found to be correlated with intensity of SEP and fertility. Larger ovum size may lead to greater distention of the infundibulum and greater SEP and ultimately a larger egg weight. Following SEP, oviductal embryonic development may begin utilization of albumen proteins and influence albumen characteristics even at lay. It is also possible that embryonic development affects egg component weights and characteristics by altering transit time of the egg through the oviduct.

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