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≪Research Note≫

Effects of Observed Incubation Behavior on Egg Production in Laying Hens of a Commercial Chicken Breed and Detection of Single-Nucleotide Polymorphisms Associated with the Incubation Behavior

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Upon contact with laid eggs, avians initiate incubation behavior and stop laying additional eggs. This phenomenon suggests that the productivity of laying hens in free-range facilities may decrease because of frequent contact with laid eggs. Here, we examined whether hens of a commercial breed exhibit incubation behavior in a free-range facility and whether egg productivity subsequently decreases. One-hour observations were performed twice weekly for 3 weeks, during which 9 of 129 hens (7.0%) exhibited incubation behavior (i.e., sitting on eggs) in the free-range facility and were defined as incubating hens. During 4 d of continuous behavioral observation, incubating and non-incubating hens laid the same number of eggs statistically (4.6 and 3.6, on average, respectively); however, incubating hens spent significantly more time on average incubating the eggs (2071.9 min) than did the non-incubating hens (20.9 min; $P<0.05$), indicating a clear behavioral difference. Subsequently, the incubation behavior and egg productivity of incubating hens and a Silkie Fowl breed hen, which is known to exhibit typical incubation behavior and cessation of laying, were continuously compared for 27 d. The average minutes spent incubating eggs during the observation period increased in both the incubating hens and Silkie Fowl hen and the total time was almost the same (18,088.5 and 23,092 min, respectively). However, the Silkie Fowl hen stopped laying on day 17 after laying 17 eggs, whereas the incubating hens continued laying throughout the observation period. Incubating hens laid an average of 24.5 eggs, indicating that some hens (at least those of the commercial breed used in our study) can continue laying while exhibiting incubation behavior. A single-nucleotide polymorphism associated with incubation behavior was detected on chromosome 4 through genome-wide association analysis.

Key words: commercial breed laying hens, egg production, incubating behavior

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Introduction

Cages that can individually house laying hens, such as conventional battery cages, are superior to other housing systems because they reduce the labor requirement for the management of hens and the space needed for rearing (Appleby *et al*., 2004). However, the expression of some innate hen behaviors, such as foraging and dust bathing, is known to be restricted in battery cages (Martin, 1987; Baxter, 1994). Considering this, rearing laying hen flocks on the flat floor of barns (i.e., free-range facilities) has been proposed as an alternative rearing system.

Hens reared in free-range facilities typically have more chances to contact laid eggs than hens reared using other rearing systems, such as battery cages, which are generally equipped with traps that collect eggs immediately after laying. Some studies have suggested that the frequent contact of hens with laid eggs in free-range facilities can lead to decreased

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egg productivity because hens might begin incubating and stop laying after such contact (Romanov *et al*., 2002; Sharp and Hocking, 2009). This hypothesis is based on the common reproductive physiology of avian species, namely, female birds continuously lay one egg per day, but this behavior ceases when a suitable number of eggs (one clutch) accumulates in the nest (Haywood, 1993; Sharp and Hocking, 2009). In this case, physical contact of the hens with laid eggs during incubation reportedly serves as a stimulus to cease laying. For instance, it has been reported that if a single egg was removed from the nest immediately after laying each day or the clutch of eggs was removed from the nest, the female continued laying or restarted laying, respectively (Haywood, 1993; Sharp and Hocking, 2009).

Research has shown that hens with a high genetic ability to lay eggs have lost the ability to incubate eggs. For example, hens of White Leghorn, a breed with a high genetic ability to lay eggs, do not exhibit incubation behavior (Romanov *et al*., 2002), whereas native breeds such as Silkie Fowl, which have never been genetically selected for egg laying, exhibit strong incubation behaviors (Jiang *et al*., 2005; Shimmura *et al*., 2010). From this perspective, laying hens of commercial breeds most commonly used for industrial egg production may not incubate their eggs because of their high genetic ability to lay eggs. However, to the best of our knowledge, no studies have examined whether laying hens of commercial breeds reared in free-range facilities exhibit incubation behavior and stop laying after contact with laid eggs.

Based on the above background, we examined whether laying hens of a commercial breed incubate their laid eggs in a free-range facility and whether such incubation behavior is associated with egg productivity. To obtain insights into the genes involved in regulating egg productivity, we conducted a genome-wide association analysis of single-nucleotide polymorphisms (SNPs) associated with incubation behavior.

Materials and Methods

Animals

A total of 68 and 61 laying hens of a commercial breed (white layers) developed for egg production in Japan were reared in a free-range facility (80.9 m^2) for 3 weeks in 2019 and 2020, respectively. Hens were 510 days old at the start of the experiment, when they laid eggs almost daily. The floor of the facility was covered with bedding, and the hens were fed normal feed for laying hens (Chubu Shiryo Co., Ltd., Aichi, Japan) and freshwater *ad libitum*. One light:dark cycle was designated as 1 d, beginning at 4:00 and ending at 20:00; the light and dark periods were 16 and 8 h, respectively. The light intensity at hen eye level during the light and dark periods was 7.5 and 0 lux, respectively. A 300-day-old Silkie Fowl hen was used as a control for laying hens. All animal experiments were approved by the Animal Care and Use Committee of Rakuno Gakuen University (DH15C6 and DH19C11).

Identification of Incubating Hens

Hen incubation behavior was observed twice per week, on Monday and Thursday, for 3 weeks. On observation days, the behavior of hens in the facility was observed for 1 h beginning at 11:00, immediately before the daily collection of eggs. The experiment was repeated twice; the first experiment began on October 24, 2019, using 68 hens, and the second experiment began on October 19, 2020, using 61 hens. Incubating hens exhibit characteristic behavior: sitting on laid egg(s) after gathering the egg(s) under their chest using their beak (Jiang *et al*., 2005; Sharp and Hocking, 2009). Therefore, we defined hens exhibiting this behavior more than twice during the observation period as incubating hens. Hens not exhibiting this behavior were regarded as non-incubating hens. For the genome-wide association analysis (described below), incubating hens (n=5) and non-incubating hens (n=63) reared in 2019 (Table S1) were used as case and non-case samples, respectively.

Measuring the Duration of Incubation Behavior

In both the 2019 and 2020 experiments, the incubating hens were housed individually in meshed-floor pens (1.5×1.5×1.5 m) immediately after identification. Pens containing hens were placed on the bedding on the animal facility floor, and the hens could contact their laid eggs freely, similar to a free-range facility. Hens in floor pens were provided with continuous free access to water and the same feed as used in the initial experiment. The animal room lighting conditions were the same as those used in the initial experiment. The behavior of each hen was recorded continually using a monitoring camera (CS-W50FHD, Planex, Tokyo, Japan) attached to the top of each floor pen. The amount of time the hens sat on more than one laid egg was measured by checking the recordings and was defined as the duration of incubation. To induce hen incubation behavior, eggs laid on the floor of the pens were not collected after initiating the hen behavior recordings. Measurement of the duration of incubation behavior using the recordings was started at 4:00 on the day after the first laid egg appeared.

The behavior of incubating hens was initially monitored for 4 d and compared with that of eight non-incubating control hens (four non-incubating hens were analyzed each year, 2019 and 2020, at the same time as when the incubating hens were analyzed). The behavior of non-incubating hens was monitored using the same procedure as that used for observing incubating hens. During this 4-d observation period, the behavior of one of the incubating hens reared in 2019 was not recorded owing to malfunction of the monitoring camera. After that period, the incubation behavior of the four incubating hens reared in 2020 continued until 27 d after the start of observation.

Because Silkie Fowl hens exhibit typical incubation behavior (Shimmura *et al*., 2010), the duration of the incubation behavior of a Silkie Fowl hen was also determined for 27 d from the start of observation using the same procedure as used for the incubating hens in 2020. Immediately before starting the experiment, the Silkie Fowl hen was kept in a cage equipped with an egg trap that did not allow the hen to contact laid eggs to prevent premature induction of incubation behavior. The duration of the incubation behavior of the Silkie Fowl hen over the 27-d period was compared with that

of the four incubating hens.

Measurement of Egg Productivity

The recordings of the incubating hens reared in floor pens for 4 d in the initial experiment were re-examined, and the number of eggs observed in the recordings was regarded as the number of laid eggs. The average number of eggs laid during the observation period was compared with the average number determined from the recordings of the eight non-incubating hens. The same procedure for determining the average number of eggs laid was used for the four incubating hens observed for 27 d from the start of the observation. The number of eggs laid by the Silkie Fowl hen during the 27-d observation period was also determined and compared with the average number of eggs laid by the four incubating hens during the 27-d observation period.

Identification of SNPs Associated with Incubation Behavior

The 68 hens reared in 2019 were used in this experiment and were examined for incubation behavior. They were designated as either incubating hens (n=5) or non-incubating hens (n=63) (Table S1), and were used as the case and non-case samples for the genome-wide association analysis. A 3-mL blood sample was collected from each hen. DNA was extracted from blood using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). A genome-wide association analysis was conducted to identify SNPs associated with incubating/non-incubating traits. Briefly, DNA was analyzed using restriction site–associated DNA sequencing (Baird *et al*., 2008; Yamashita *et al*., 2020). The resulting reads were preprocessed using Trimmomatic (ver. 0.36) with the following parameters: ILLUMINACLIP Tru Seq3-PE-2.fa: 2:40:15, LEADING: 20, TRAILING: 20, SLIDINGWINDOW: 4:20, and MINLEN: 36. After pre-processing, the remaining reads were aligned to the chicken reference genome (GRCg6a), which was downloaded from Ensemble (https://m.ensembl.org/Gallus_gallus/Info/Annotation#assembly) using BWA (ver. 0.7.15), and the SNPs were then called using Stacks (ver. 2.5.4). As a result, 151,657 SNPs were called and filtered using the following thresholds: call rate within a locus $>$0.5 and minor allele frequency $>$0.05 (Yamashita *et al*., 2020). The minimum number of samples in which the site must have been scored to be included in the filtered dataset was set to 30 of 68 samples. After filtering, 24,877 SNPs remained, and their association with the incubation trait was analyzed. The genetic structure of the 68 hens used for the genome-wide association analysis was examined using principal component analysis of the SNP dataset using Tassel (ver. 5.0) (Bradbury *et al*., 2007; Smitz *et al*., 2018). Briefly, the principal components indicate directions with the highest variance. Accordingly, the eigenvalues were calculated for all principal components. A mixed linear model of Tassel (ver. 5.0) (Bradbury *et al*., 2007) was used for the association analysis with a kinship matrix as the cofactor to avoid spurious associations owing to relatedness and population structure (the Q+K method). Specifically, the statistical model can be described based on Henderson's notification (Henderson, 1975) as follows:

$$\mathbf{y}=\mathbf{X\beta}+\mathbf{Z\mu}+\mathbf{e},$$

where $\mathbf{y}$ is the vector of observations, $\boldsymbol{\beta}$ is an unknown vector containing fixed effects including genetic markers and population structure (Q), $\boldsymbol{\mu}$ is an unknown vector of random additive genetic effects from multiple background quantitative trait loci for individuals or lines, $\mathbf{X}$ and $\mathbf{Z}$ are the known design matrices, and $\mathbf{e}$ is the unobserved vector of random residuals. Each marker allele was fit as a separate class, with heterozygotes fit as additional marker classes. The resulting marker effect is not decomposed into additive and dominance effects, but is simply tested for overall significance. The $\boldsymbol{\mu}$ and $\mathbf{e}$ vectors are assumed to be normally distributed, with a null mean and variance of

$$\mathrm{Var}\begin{pmatrix}\boldsymbol{\mu}\\ \mathbf{e}\end{pmatrix}=\begin{pmatrix}\mathbf{G} & 0\\ 0 & \mathbf{R}\end{pmatrix}$$

where $\mathbf{G}=\sigma_\alpha^2\mathbf{K}$ with σ_α^2 as the unknown additive genetic variance, and $\mathbf{K}$ is the kinship matrix. Tassel provides a function for estimating $\mathbf{K}$ from a set of random markers covering the whole genome. (Hardy and Vekemans, 2002; SAS Institute, 2003). Homogeneous variance is assumed for the residual effect, that is, $\mathbf{R}=\mathbf{I}\sigma_e^2$, where σ_e^2 is the unknown residual variance. The estimates σ_α^2 and σ_α^2 of the restricted maximum likelihood estimation are obtained using the expectation and maximization algorithm (Laird and Ware, 1982). In this analysis, a binary phenotype (incubating/non-incubating behavior identified in 2019, Table S1) was applied. The mixed linear model used here can be applied to a binary phenotype (Cook *et al*., 2017). Q-Q plots were applied to the P-values obtained from the association analysis using Tassel 5 (Bradbury *et al*., 2007; Voorman *et al*., 2011).

The Manhattan plot was drawn using Tassel (ver. 5.0). The significance level for assessing associations was modified according to Bonferroni's correction (Bonferroni, 1935), and the significance level for detecting the association of one SNP with the trait, 0.05, was divided by the number of SNPs analyzed, 24,877 ($=2.0\times10^{-6}$). Because hens have heterologous sex chromosomes (Z and W), the association of SNPs on the sex chromosomes with the trait could not be analyzed together with SNPs on autosomal chromosomes. Thus, analyses of the sex chromosomes were omitted from the experiment.

Statistical Analyses

Data from the experiments were analyzed using Student's t-test. Detailed explanations of the statistical analyses are provided in the respective figure legends or notes for the respective tables.

Results

Identification of Incubating Hens

The proportion of incubating hens among the total number of hens examined was the same in both 2019 and 2020 (7.4% and 6.6%, respectively); on average, 7.0% of the total hens examined were determined to be incubating hens (Table S1).

Persistence of Incubation Behavior among Incubating Hens

In our experiment, incubating hens were identified by observing their behavior for 1 h twice a week. Thus, it is possible that the incubation behavior of the incubating hens occurred by chance and therefore did not differ statistically from that of non-incubating hens (Table S1). To exclude this

possibility, we compared the duration of incubation behavior of incubating hens with that of non-incubating hens for 4 continuous days. As both incubating and non-incubating hens laid one egg almost every day, the average number of eggs laid during the 4-d observation period was 4.6±0.2 and 3.6±0.5, respectively (Fig. 1A and Table S2). Thus, both types of hens were exposed to a sufficient number of laid eggs to exhibit incubation behavior during the observation period. However, the average duration of incubation behavior for the incubating hens was 2,071.9±306.5 min, which was significantly longer than that of the non-incubating hens, 20.9±12.5 min (Table S2, $P<0.05$). To assess the persistence of the incubation behavior over a period longer than 4 d, the duration of incubation behavior for four incubating hens was monitored until day 27 and compared with that of a Silkie Fowl hen, a breed known to exhibit typical incubation behavior (Shimmura *et al*., 2010). Both the incubating hens and the Silkie Fowl hen laid one egg almost every day, laying 24.5±1.7 and 17

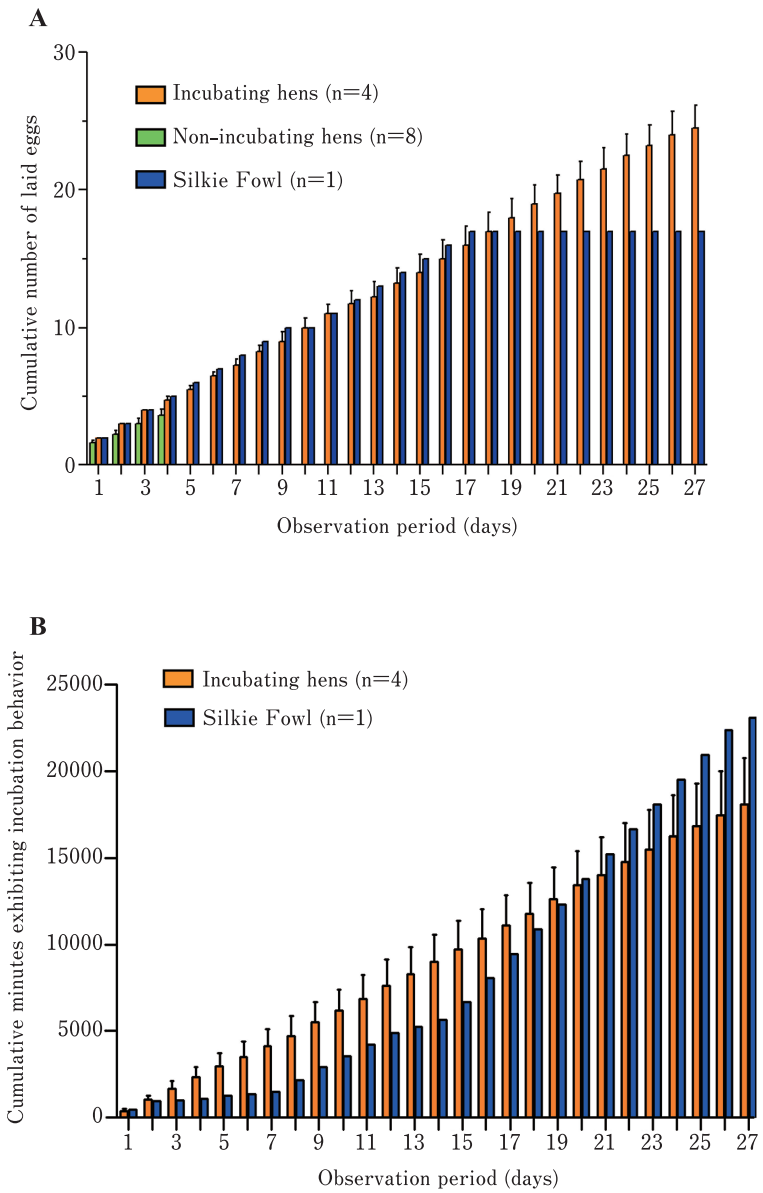

Fig. 1. **A: Cumulative number of eggs laid by the hens during the 27 d of observation.** Note that the observation period of non-incubating hens was 4 d, and that of the incubating hens and the Silkie Fowl hen was 27 d. Error bars denote standard error of the mean. "n" denotes the number of hens used for the analysis. **B: Cumulative minutes exhibiting incubation behavior by the hens during the 27 d of observation.** Error bars denote standard error of the mean. "n" denotes the number of hens used for the analysis.

Table 1. **Duration of incubation behavior and number of eggs laid by four incubating hens identified in a previous 1-h observation experiment in the free-range facility and one Silkie Fowl hen reared in individual floor pens for 27 days**

Type of hen	Hen ID	Duration of incubation behavior (min)	Number of eggs laid
Incubating	D102	24,094	20
Incubating	D146	18,606	28
Incubating	D185	11,065	25
Incubating	D198	18,589	25
Average±SEM		18,088.5±2,675.7	24.5±1.7
Silkie Fowl	—	23,092	17

SEM denotes the standard error of the mean. Measurement of the duration of incubation behavior was initiated the day after the first laid egg appeared and continued for 27 days. The total number of eggs laid on the last day of the measurement period is shown as the number of eggs laid. In the floor pens, the hens could access the laid eggs at all times, just as if they were in a free-range facility.

eggs, respectively, during the observation period. Thus, both types of hens were exposed to a sufficient number of laid eggs to exhibit incubation behavior during the observation period (Fig. 1A and Table 1). The average duration of incubation behavior exhibited by the incubating hens over the 27-d observation period was 18,088.5±2,675.7 min, which was slightly shorter than that of the Silkie Fowl hen (23,092 min, Table 1). Although the profile was marginally different, the cumulative number of minutes exhibiting incubation behavior increased continuously throughout the observation period in both the incubating hens and Silkie Fowl hen (Fig. 1B) such that all hens incubated the laid eggs constantly every day.

Egg Productivity of Incubating Hens

During the 4-d observation period, an average of 4.6±0.2 and 3.6±0.5 eggs were obtained from each of the incubating and non-incubating hens, respectively; the difference was not statistically significant (Table S2). Steen and Parker (1981) and Sharp and Hocking (2009) reported that hens stop laying after laying 10 to 20 eggs in the nest. Consistent with these studies, the number of eggs laid by the Silkie Fowl hen over the 27-d observation period was 17, and this hen did not lay additional eggs after day 17 (Table 1 and Fig. 1A). During the 27 d of observation of the incubating hens, the average number of eggs laid was 24.5±1.7, and the hens laid eggs throughout the observation period (Table 1 and Fig. 1A).

Identification of SNPs Associated with Incubation Behavior

Some genetically different individuals were identified among the 68 analyzed hens (Fig. S1A); however, the Q-Q plot indicated that the bias associated with the genetic structure of the hens used in the experiment had been sufficiently eliminated (Fig. S1B) (Bradbury *et al*., 2007; Smitz *et al*., 2018). An SNP that was significantly associated with the incubation trait ($P=1.6\times10^{-6}$) was identified on chromosome 4 at position 76542731 (Fig. 2 and Table S3).

Discussion

In this study, some of the commercial-breed hens exhibited incubation behavior in a free-range facility in which the hens could come into contact with laid eggs, but no subsequent cessation of laying occurred.

In our study, the duration of incubation behavior exhibited by the incubating hens was comparable to that of a hen of the Silkie Fowl breed (Table 1 and Fig. 1B). As we analyzed only one Silkie Fowl hen in this study, the data might not represent the precise incubation duration usually observed in this breed (Shimmura *et al*., 2010). However, breeds exhibiting normal incubation duration, such as the Bantam breed, usually exhibit the same duration of incubation as was observed with the Silkie Fowl hen and incubating hens in our study (Lea *et al*., 1981; Sharp and Hocking, 2009). Thus, the incubation duration observed in the incubating hens was comparable to that of breeds exhibiting normal incubation behavior.

In addition to persistent incubation behavior, the Silkie Fowl hen and incubating hens exhibited some characteristics specific to hens during incubation, such as nest building (collecting bedding material and laying eggs at one place on the floor pen) (Duncan and Kite, 1989) and the formation of a brood patch (de-feathering of the chest area that is in contact with the eggs) (Book *et al*., 1991; Sharp and Hocking, 2009). These phenomena were not observed in non-incubating hens. Commercial breeds are typically produced by crossing three or four breeds; with three to four breeds used as grandparents. Thus, it can be considered that breeds exhibiting different incubation behaviors were used as the great-grandparents of the commercial breed used in this study; therefore, individuals exhibiting different incubation behaviors (Table S1) were observed among the commercial breeds used in our study.

Although the incubating hens exhibited incubation behavior comparable to that of breeds known to exhibit normal incubation behavior (Lea *et al*., 1981; Sharp and Hocking, 2009), the incubating hens did not stop laying after the number of eggs that were sufficient to induce the cessation of laying (e.g., 10 to 20 eggs) had accumulated in the floor pen (Table 1 and Fig. 1A) (Steen and Parker, 1981; Sharp and

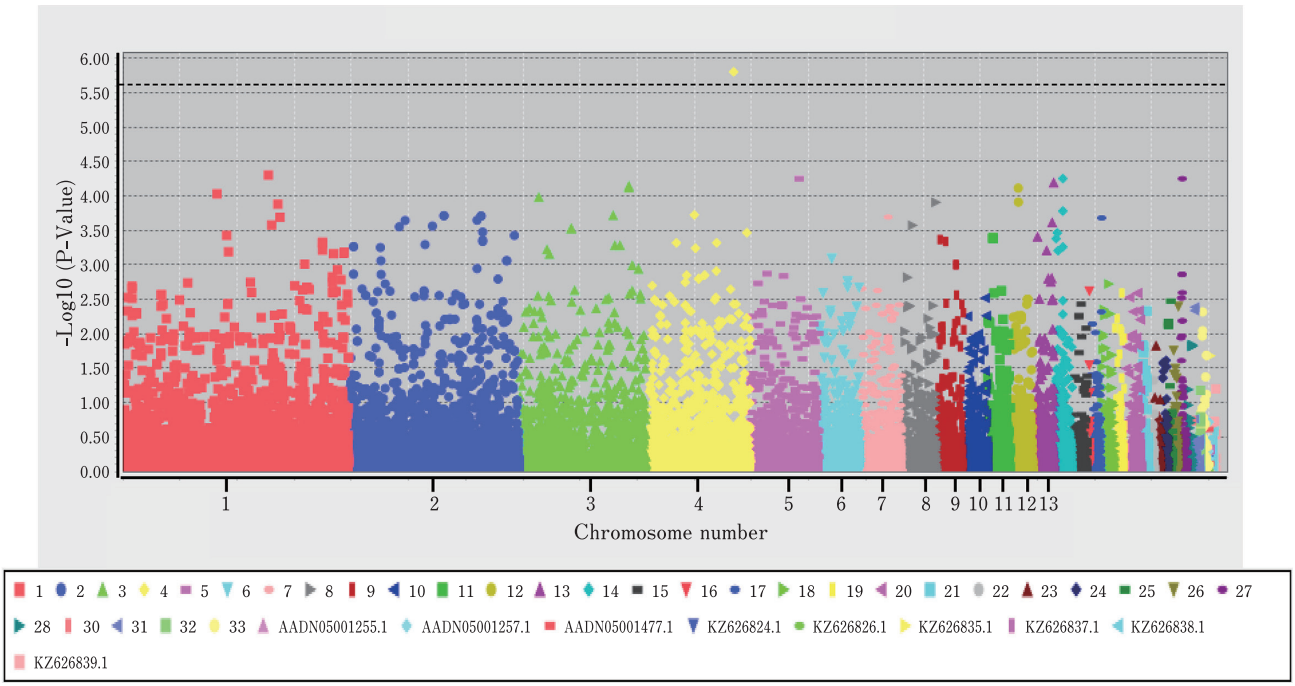

Fig. 2. **Results of genome-wide association analysis of incubation trait.** Manhattan plots depict the results of a genome-wide association analysis of the incubation trait. Each number on the x-axis denotes a chromosome. Chromosome numbers after chromosome 13 were omitted from the x-axis. In the reference genome, regions that did not map onto the chromosomes are described by IDs (letters and numbers) in the box. Sex chromosomes were not analyzed for the reasons described in the Materials and Methods section. On the y-axis, *P*-values are shown as –log10(*P*). Genome-wide association analysis was performed for the hens reared in 2019 (Table S1). For the analysis, incubating hens ($n=5$) and non-incubating hens ($n=63$) were applied as case samples and non-incubated hens, respectively.

Hocking, 2009). Incubation behavior and cessation of laying generally occur together (Haywood, 1993; Sharp and Hocking, 2009); however, our results indicate that they can occur separately. Changes in molecular or hormonal mechanisms are thought to be related to the observed separation of incubation behavior and cessation of laying; however, the details remain to be elucidated.

The rate of change in the cumulative minutes in which incubation behavior was exhibited was almost the same every day in the incubating hens; however, in the Silkie Fowl hen, the rate increased after approximately day 17 (Fig. 1B). Although our data (Fig. 1B) were derived from only one Silkie Fowl hen and thus might not be reliable, it has been reported that the amount of time incubation behavior is exhibited for increased after one clutch of eggs had been laid in hen breeds exhibiting normal incubation behavior, such as the Bantam breed (Steen and Parker, 1981; Sharp and Hocking, 2009). The profile of the Silkie Fowl hen (Fig. 1B) is consistent with these reports. The same tendency was not observed in the incubating hens (Fig. 1B), perhaps because of the inability of the hens to perceive that the number of eggs laid was sufficient to induce an increase in the incubation time. However, the incubating hens were exposed to laid eggs in the free-range facility for 3 weeks before measurement of the duration of incubation was initiated for 27 d. It is therefore possible that incubating hens might have already started to increase the amount of time spent incubating by the start of the 27-d period of measuring the duration of incubation, without stopping laying.

Incubation reportedly stimulates the subsequent cessation of laying (Haywood, 1993; Sharp and Hocking, 2009). Thus, the SNP identified as being associated with the incubation trait in our study could be associated with egg productivity in breeds other than those used in our study. Therefore, we attempted to identify the SNPs associated with the incubation trait. In our study, an SNP on chromosome 4 was identified to be associated with the incubation trait (Fig. 2 and Table S3). This SNP was located in a region that contained a non-coding RNA (XR_005859184.1) but no genes, and a gene (cytoplasmic polyadenylation element binding protein 2) was located at 82700 and 192700 bp upstream of the SNP. Few studies have examined the association between specific gene/DNA regions and incubation behaviors. Jiang *et al*. (2005) and Shen *et al*. (2012) identified DNA polymorphisms associated

with the incubation trait on chromosomes 2 and 5, respectively, rather than chromosome 4, as was the case in our study. Because those studies used different breeds (Blue-shell and Ningdu Sanhuang, respectively), this discrepancy could indicate that the gene/DNA region associated with incubation behavior differs between breeds.

Genome-wide analyses to identify SNPs associated with particular traits are usually conducted using large numbers of samples (i.e., more than hundreds) to avoid false-positive SNP identification. Thus, the SNP identified on chromosome 4 (Fig. 2 and Table S3) from the analysis of only 68 samples in our study could be a false positive; therefore, the results of our genome-wide analysis should be verified in the future using a large number of samples. However, in the case of traits regulated by one dominant SNP, such as the pace trait in horses (Andersson *et al*., 2012), the associated SNP can be clearly detected even if the sample number is small (e.g., n=127) (Amano *et al*., 2018). As such, the SNP identified on chromosome 4 could be considered a good candidate SNP associated with the incubation trait.

Based on behavioral observations of 2019, we used five incubating hens as cases and 63 non-incubating hens as non-cases in our study to identify SNPs associated with incubation behavior (Table S1). As the ratio of the number of case samples (the number of incubating hens) and the number of non-case samples (the number of non-incubating hens) used to identify SNPs associated with the incubating trait was biased, again, it is possible that the SNP identified on chromosome 4 in our study was a false positive (Fig. 2). However, several previous genome-wide association analyses have identified loci related to brooding traits on chromosome 4 (Johnsson *et al*., 2016a, b). Thus, these reports support our findings.

In summary, we found that commercial breed hens (white layers) can exhibit incubation behavior in a free-range facility in which the hens can contact laid eggs, but the hens did not subsequently stop laying in our study. This may indicate that careful selection of the breed used for laying could prevent a decrease in egg productivity by hens in free-range facilities, even if they exhibit incubation behavior. An SNP associated with the incubation trait was identified on chromosome 4 in the commercial breed used in our study. The detected SNP could be associated with egg productivity in breeds other than those used in our study.

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Conflicts of Interest

The authors declare no conflict of interest.

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