New-born chicks are vulnerable to bacterial infections and not good at regulating body temperature. There is a close relationship between thermal regulation and immunity, however, the underlying mechanism is not well understood. *Salmonella Pullorum* (SP) is a major concern in developing countries and causes significant economic losses in poultry industry. Early body temperature (EBT) has previously shown to be correlated with host immunity and resistance to pullorum disease. In this study, we challenged three independent chick populations (Beijing You, Dwarf and Rhode Island Red) with SP at 4 days of age, and rectal temperature was measured before and after the SP attack from 2 to 7 days of age. Host defense to SP was evaluated by survival and spleen SP carrier status. The results showed that chicks with higher EBT before SP infection tend to have higher resistance to later SP attack in two populations (Dwarf and Beijing You). The association between EBT before SP attack and SP resistance was non-significant in Rhode Island Red population \( (P = 0.06) \), but the trend was consistent with the other two populations. We also found low to moderate heritability in all three populations for EBT before and after the SP attack ranging from 0.14 to 0.20. Genome-wide association studies identified several genomic regions and biological pathways determining EBT before SP attack, which provides candidate functional genes of this trait. Our results reveal the genetic determination of EBT, and the relationship between EBT and SP resistance, providing an alternative strategy for improving SP resistant activities in chicken.

**Keywords:** *Salmonella Pullorum*, body temperature, disease resistance, heritability, genome-wide association study, chicken
INTRODUCTION

Pullorum disease, an acute and systemic chick infectious disease caused by Salmonella Pullorum (SP), occurs frequently and is a major economic concern for chicken farms in developing countries (Barrow et al., 2012). SP can cause high mortality rates in chicks under 20 days old and yet few symptoms in adult chickens. In the poultry industry, disease resistance is an important trait with substantial economic value and disease control is drawing more and more public concerns (Cheng et al., 2013). Existing disease management is not enough to prevent disease outbreaks and genetic selection for resistant birds has been regarded as a promising complementary strategy. Difficulty in measuring disease resistance phenotypes remains a major obstacle in genetic research and breeding. This difficulty arises since it is demanding and costly to do large-scale challenge tests in farm settings. Additionally, different infection models have been used in the study of Salmonella resistance, complicating the interpretation of results (Calenge et al., 2010). Despite these problems, it is important to identify traits associated with SP resistance to aid in breeding and selection strategies.

Body temperature is an important physiological trait and indicator of health status (Sund-Levander et al., 2002). Young animals are unable to regulate their own body temperature and generally susceptible to various pathogen infections. There is a pronounced rise in the body temperature of chicks during the first week after hatching, particularly during the first four days (Lamoreux and Hutt, 1939). There are also breed differences of thermoregulation in newly hatched chicks (Dunnington and Siegel, 1984), indicating a genetic determination of early body temperature (EBT). Interestingly, chicken resistance to SP has been shown to be positively correlated with rapid rise of body temperature in the first 10 days post-hatch (Roberts and Cards, 1935). A two-generation selection experiment further confirmed the association between EBT and SP resistance (Hutt and Crawford, 1960a), suggesting the possibility of breeding resistant chicks based on EBT instead of pathogen exposure. In fact, many studies have reported the close relationship between body temperature and immunity regulation (Hori et al., 1991; Watanabe et al., 2008; Fisher et al., 2010; Morrison and Nakamura, 2019). However, the genetic basis of EBT has not been systematically investigated and its correlation with resistance to SP needs more detailed demonstration.

In the present study, we orally challenged three independent chicken breeds/lines with SP at 4th day post-hatch, including a highly selected commercial line (Rhode Island Red, RIR), a Chinese local line (Beijing You, BY), and a synthetic layer line (dwarf, DW) to achieve disease phenotypes. We recorded the body temperature of chicks before and after the SP challenge to better understand the relationship between EBT and SP resistance. The differential analysis among different resistance groups was carried out using PROC GLM of SAS (version 9.2; SAS Institute Inc., Cary, NC). For our genetic analysis, SP resistance was defined as a three-state categorical trait (dead, carrier, clear), and temperature as a continuous trait. Variance and covariance components were estimated using the DMU software package (Madsen and Jensen, 2008). Bivariate analyses of resistance-EBT were performed using a bivariate threshold model, which was fitted using the RJMC module in the DMU software package.

**Abbreviations:** BY, Beijing You; DW, Dwarf Chicken; EBT, Early body temperature; GWAS, genome-wide association study; LD, linkage disequilibrium; LMM, linear mixed model; PCA, principle component analysis; RIR, Rhode Island Red; SNP, single-nucleotide polymorphism; SP, Salmonella pullorum.

**MATERIALS AND METHODS**

**Ethics Statement**

All experiments were approved by the Animal Care and Use Committee of China Agricultural University (Approval ID: XXCB-20090209). All the animals were fed and handled according to the regulations and guidelines established by this committee, and all efforts were made to minimize suffering.

**Chickens and Bacterial Challenge**

The details about the chicken populations and challenge test procedure can be found in (Li et al., 2018). Briefly, 621 DW, 586 RIR, and 482 BY chicks were orally inoculated with 4.8×10⁷ CFU of SP culture at 4 days post-hatch. 40 chicks from each line were randomly selected as control group, which was mock-challenged with the same volume (0.5 mL) of phosphate buffer saline. After 36 days, all chicks still alive were killed by neck dislocation, and their spleens were removed aseptically and immediately plated onto MacConkey agar media with an inoculation loop in order to check for the presence of SP. Chicks were classified as either susceptible (dead), carriers (with detectable SP load in the spleen) or clear (without detectable SP load in the spleen).

**Body Temperature Determination**

Chick temperature was estimated with clinical electronic thermometers. Thermometer probes were dipped in glycerin to facilitate insertion into the cloaca, and held in the chick rectum at 1.5 cm for 15 s before recording temperature. All temperature measurements were conducted between 13:00-16:00 from 2 to 7 days post-hatch. Early body temperature (EBT) was defined as the average temperature of 2 to 4 days post-hatch, because chick body temperature experiences a dramatic rise during the first week after hatching (Lamoreux and Hutt, 1939; Hutt and Crawford, 1960a). Temperature measurement at 4 days post-hatch was taken before SP inoculation. Fever was determined as the average temperature of 5 to 7 days.

**Heritability Analyses**

Chicks in each population were designated as either susceptible, carrier or clear, which represents different degrees of SP resistance. The differential analysis among different resistance groups was carried out using PROC GLM of SAS (version 9.2; SAS Institute Inc., Cary, NC). For our genetic analysis, SP resistance was defined as a three-state categorical trait (dead, carrier, clear), and temperature as a continuous trait. Variance and covariance components were estimated using the DMU software package (Madsen and Jensen, 2008). Bivariate analyses of resistance-EBT were performed using a bivariate threshold model, which was fitted using the RJMC module in the DMU software package.
Heritabilities of EBT were estimated with AI-REML in a linear model of the DMU software package. Both models included the fixed effect of the cage and the random effects of additive genetics, as shown in matrix notation:

\[ y = X\beta + Z\mathbf{a} + e \]

where \( y \) is a vector of phenotypic value; \( \beta \) and \( \mathbf{a} \) are the vectors of fixed effects and random additive effects, respectively; \( X \) and \( Z \) are appropriate incidence matrices of fixed effects and random additive effects, respectively; and \( e \) is the random residuals.

**DNA Isolation, Genotyping and Quality Control**

We isolated individual genomic DNA from blood or muscle samples by the classical phenol-chloroform procedure. The DNA integrity was verified by agarose gel electrophoresis and the purity was checked by A260/280 ratio using the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific™). Not all the chicks were used for genotyping. Only the individuals with conclusive symptom and high DNA quality were genotyped. Some individuals in the clearance group were also randomly abandoned to match the corresponding phenotype proportion. A total of 842 samples were sent for genotyping. A total of 842 qualified individual genomic DNA samples were genotyped using the Affymetrix 600 K chicken high-density array (Affymetrix, Inc. Santa Clara, CA, USA).

The software Axiom Analysis Suite 3.1 was used for single-nucleotide polymorphism (SNP) calling and initial quality control with the Best Practices Workflow setting from the raw data in the form of CEL files. Only samples with dish quality control (DQC) of 0.82 or better and call rate >95% were used for the downstream analyses. The SNP QC metrics were set to default values recommended by Affymetrix, except that only “PolyHighResolution” SNPs were used in our analysis. Furthermore, we excluded SNPs with unknown or repeated physical positions with an R script. SNPs on sex chromosomes were also discarded because the current statistical methods are not powerful enough to detect association between phenotypes and sex-related genotypes. After these QC steps, 818 samples and 452,291 SNPs remained. To increase the power of association analysis, we dropped 85,140 SNPs due to low variation among subpopulations (monomorphic in any one of the three breeds), 62,450 variants deviating from Hardy-Weinberg equilibrium (HWE) test (\( P < 1 \times 10^{-5} \)) and 1,774 SNPs with minor allele frequency (MAF) < 0.05 using PLINK v1.90 (Purcell et al., 2007). Missing genotypes were imputed based on information of the remaining SNP genotypes from each subpopulation separately, according to the software Beagle Version 4 (Browning and Browning, 2009). A total of 818 samples and 302,927 SNPs were included in the subsequent genome-wide association analysis. The detailed sample composition used in the GWAS is presented in the Supplementary Table 1.

**Candidate Genes and Functional Annotation**

The gene position information was annotated using BioMart in the Ensembl database (Ensembl Genes 96). Candidate genes were retrieved within 1 Mb of the significant SNPs. To provide insight into the functional enrichment of candidate genes, we carried out GO (Gene Ontology) and Pathway analysis using Metascape (Zhou et al., 2019). Metascape is an integrated portal that
combines functional enrichment, interactome analysis, gene annotation, and membership search to leverage over 40 independent databases. As the knowledge base for human is the most comprehensive, we converted the chicken genes to their human orthologs for enrichment analysis.

RESULTS

Descriptive Statistics of Phenotypes
Mortality rates after SP inoculation were 25.1% for RIR, 8.3% for BY and 22.7% for DW. For RIR, 17.9% of chicks were classed as carriers, compared with 0.6% for BY and 15.8% for DW. Finally, 57% of RIR completed cleared the infection, compared with 91.1% for BY and 61.5% for DW.

Normal temperature variation of the control group from 2 to 7 days post-hatch is shown in Figure 1. EBT increased over time in all three lines, and is particularly sharp during the first two days of measurement, consistent with earlier studies (Hiitt and Crawford, 1960b). At 7 post-hatch, the average temperatures of three populations were not significantly different. The mean, standard deviation (SD), coefficient of variation (CV), minimum and maximum values of average early temperature are shown in Table 1. We found that the average EBT for DW chicks was 0.3–0.4°C less than the other two lines (p-value < 0.01).

Correlations Between EBT and SP Resistance
Differences in EBT for chicks with different SP outcomes is shown in Figure 2. After infection, EBT for challenged chicks increased significantly compared to the control group (p-value < 0.01).

As shown in Figure 3, we found that average EBT for SP-susceptible birds was lower in all three chicken lines compared to carrier and resistant birds (p < 0.05 in DW and BY). EBT was also lower for RIR; however, the results were not statistically significant (p-value = 0.06). There was no significant difference in EBT between carrier and clear birds (p-value > 0.05).

Heritability and Genetic Correlations
Genetic parameters are important references for the selection of infection-resistant animals in breeding. Higher heritability means that the trait could be improved through direct phenotype selection. Most disease trait showed heritabilities lower than 0.1, which means the selection could not work very well. However, we can use other traits to carry an indirect selection. The estimated heritability of EBT is shown in Table 2. EBT before and after SP infection shows low to moderate heritability (0.14–0.20). The genetic correlation between resistance and EBT is 0.23 in DW, 0.09 in RIR and 0.04 in BY, however the reduced correlation in BY is likely due in

### TABLE 1 | Early body temperature in three study lines.

| Line | Mean* | SD  | CV  | Min  | Max  |
|------|-------|-----|-----|------|------|
| RIR  | 40.37a| 0.31| 0.78| 39.20| 41.07|
| DW   | 39.92b| 0.38| 0.95| 38.90| 41.00|
| BY   | 40.24a| 0.31| 0.77| 39.13| 41.03|

*The same letter at the upper right represents no significant difference (p-values > 0.05), and different letters represents significant difference (p-values < 0.01) between the means.
FIGURE 2 | Early body temperature variation before and after salmonella infection. Chicks were challenged with SP at 4 days of age.

FIGURE 3 | Boxplots of temperature from different Salmonella Pullorum resistance; P represents the p-value between Dead and Clear groups.
large part to the low overall mortality in this population. Our results suggest that selection for increased early EBT in some populations would be a useful tool to increase SP resistance.

**Genome-Wide Association Studies**

Since all of the GWASs were carried out within a pure breed population, the population stratification was not a major confounding effect in our study. The inflation factors ($\lambda$) were estimated to be 0.9996, 1.0267 and 1.0093 in BY, DW and RIR respectively. The Manhattan plot and the Q-Q plot for the GWAS results are displayed in Figure 4. In the BY population, the single-marker association analysis identified 2 SNPs above the genome-wide significant threshold on chromosome 15. In the DW population, 3 SNPs crossing the suggestive genomewide significant threshold were identified on chromosomes 2 and 4. However, no significant SNP was identified in the RIR population. Details of the significant markers identified are presented in Table 3. One of the five SNPs are upstream SNP, and the other four are intron SNP.

**Functional Annotation of Significant Regions**

There were three significant regions detected to be associated with EBT in total. With the help of BioMart in the Ensembl database, we achieved 162 genes located within 1 Mb of the significant SNPs. These gene IDs were converted into their human orthologs, and we finally obtained 118 gene IDs which were sent to Metascape for enrichment analysis. GO and Pathway analysis were performed to determined the biological functions of these genes. 57 GO biological processes were detected, such as oxidative phosphorylation, ubiquitin-dependent protein catabolic process and MAPK family signaling cascades. Seven KEGG pathway were found, including Alzheimer’s disease, Arginine and proline metabolism, Amyotrophic lateral sclerosis (ALS) Oxidative phosphorylation, Parkinson’s disease, Non-alcoholic fatty liver disease (NAFLD), and Cardiac muscle contraction. Five

| Lines | Traits | Heritability | SE  |
|-------|--------|-------------|-----|
| DW    | EBT    | 0.178       | 0.074|
|       | Fever  | 0.200       | 0.086|
| RIR   | EBT    | 0.144       | 0.033|
|       | Fever  | 0.159       | 0.054|
| BY    | EBT    | 0.137       | 0.071|
|       | Fever  | 0.136       | 0.081|

**FIGURE 4** | Manhattan plots and Q-Q plots of GWAS results.
Reactome gene sets were identified, including MAPK family signaling cascades, Neddylation, S Phase, MAPK6/MAPK4 signaling and Platelet homeostasis. Heatmap of top 13 clusters with their representative enriched terms is presented in Figure 5.

DISCUSSION

Thermal regulation is a basic physiological process in response to infection, which has been conserved in warm-blooded and cold-blooded vertebrates’ evolution for more than 600 million years (Evans et al., 2015). A fever response is usually beneficial for the host survival during infection, and young animals show different body temperature regulations and disease resistance. It is easy to associate these two traits which may share some genetic determination.

There has been a significant interest in the genetic basis of Salmonella resistance (Berthelot et al., 1998; Beaumont et al., 1999; Girard-Santosuosso et al., 2002). In the current study, we found major differences in genetic correlations between EBT and SP resistance among different chicken populations, implying that genetic background has an important influence in these traits. In both DW and BY chickens, we observe that higher EBT tends to be associated with greater SP resistance, consistent with previous research (Hutt and Crawford, 1960b). Differences in EBT could reflect the transition rate from poikilothermy to homiothermy in the chick, and may be related to better control of thermoregulation. Alternatively, the relationship between EBT and SP resistance may be related to immune defense mechanisms such as phagocytosis, bacteriolysis or the production of antibodies (Scholes, 1940).

We also provide a detailed description of host temperature dynamics following salmonella infection in chickens. The DW chicks with the sex-linked dwarf allele (dw) showed a lower EBT than the normal chicks, which is consistent with Dunnington’s result (Dunnington and Siegel, 1984). We found evidence of increased temperature in all populations after inoculation. After challenge, susceptible birds showed significantly greater EBT than carrier or resistant birds, suggesting that they suffered more severe infection. This is somewhat unexpected, as fever is typically associated with bacterial infection and has been shown to increase host survival (Kane and Peterson, 1976; Kluger et al., 1975). However, an acute body weight loss occurred as anticipated. Besides, we observe that both clear and carrier chicken showed longer sustained elevated EBT after infection, in contrast to susceptible chicks which showed rapid increases in EBT initially, with a decrease two days post-infection.

For the first time, we estimated the heritability of early body temperature. As showed in Table 2, EBT is a trait with low to moderate heritability, indicating genetic determination plays an important role. GWAS remains a promising method for studying genomic architecture of complex traits. Our study identified 4 significant SNPs associated with early body temperature in total. However, the significant regions do not overlap among different chicken breeds, showing that the genetic composition of EBT is relatively complex just as many disease traits. The most significant SNP, Affx-50682328 is located at the intron of OSBP2 gene on GGA15. The annotation of OSBP2 (oxysterol binding protein 2) showed it has the function of cholesterol binding and lipid transporter activity. Other positional candidate genes include several Class I MHC mediated antigen processing and presentation genes such as FBXO21, FBXO32, and FBXW8; immune-related genes such as CARD8, DTX1, and TRIB1; RNA processing genes such as SF3A1, ZMAT5, DDX54. In mice study, FBXO21 was found to facilitate Lys29-linkage and activation of ASK1 (apoptosis signal-regulating kinase 1) and promote type I interferon production upon viral infection (Yu et al., 2016). Genetic variants in CARD8 gene have been reported to be associated with many infectious diseases such as inflammatory bowel

![Heatmap of top 13 clusters with their representative enriched terms.](FIGURE 5)
disease (McGovern et al., 2006), Crohn’s disease (Roberts et al., 2010) and tuberculosis (Pontillo et al., 2013). TRIB1 was reported to play a critical role in differentiation of tissue-resident M2-like macrophages (Satoh et al., 2013). These immune-related genes are very likely to connected early body temperature and SP resistance. In another chicken body temperature GWA study, Van Goor identified QTL for body temperature on GGA 14, 15, 26, and 27 (Van Goor et al., 2015). Though there are no overlapped genomic regions, our study shared a candidate gene (DDX54) with theirs (DDX42), both of which are putative RNA helicases and implicated in a number of cellular processes involving alteration of RNA secondary structure such as translation initiation, nuclear and mitochondrial splicing, and ribosome and spliceosome assembly (Linder, 2006). Interestingly, RNA splicing was recently found to be correlated with body temperature (Preußner et al., 2017), pointing out that candidate genes with similar functions deserve further research.

The present study focused on investigating the genetic determination of EBT, the correlation with SP resistance and providing positional candidate genes. More researches are necessary to explore the biological processes and molecular mechanism of these genes to associate immune activities and body temperature regulation.

CONCLUSION

In conclusion, our study describes early body temperature variation before and after SP infection. Controlling for genetic background, we show that elevated EBT is associated with greater SP resistance in BY population, and that EBT and SP resistance are correlated in the BY and DW population. Our results could prove useful in designing breeding programs for improvement of SP resistance in some chicken breeds, and provide positional candidate genes for genetic determination of early body temperature.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in FigShare https://figshare.com/articles/EBT-GWAS_zip/10059092.

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ETHICS STATEMENT

The animal study was reviewed and approved by Animal Care and Use Committee of China Agricultural University.

AUTHOR CONTRIBUTIONS

LQ, ZN and YJ conceived and designed the research. XLi performed the experiments, analyzed, and interpreted the data, and wrote the manuscript. CN, YJ and YL performed the experiments and interpreted the data. YC, XLv, LW, JZ, WY, CZ and KL interpreted the data and contributed reagents and materials.

FUNDING

This work was supported by the earmarked fund for the Beijing Innovation Team of the Modern Agro-industry Technology Research System (BAIC04–2018, BAIC04–2019), National Natural Science Foundation of China (31772581) and Chinese Agricultural Research System (CARS-41).

ACKNOWLEDGMENTS

We gratefully acknowledge our colleagues in the Poultry Team at the National Engineering Laboratory for Animal Breeding of China Agricultural University, for their assistance on sample collection and helpful comments on the study, and Judith Mank from University of British Columbia, for her kind help on the polish of the manuscript. We thank Beinongda Technology co., LTD, for their kind help in the preparation of chicken populations.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2019.01287/full#supplementary-material

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Conflict of Interest: Author CZ was employed by company Beiningdonga Tech-nology Co., Ltd, China.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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