IN SILICO TRANSCRIPTION FACTOR ENRICHMENT ANALYSIS SUGGESTS THAT CCAAT/ENHANCER BINDING PROTEINS IN THE FOLLICLES OF HYBRID HENS MAY INFLUENCE HETEROSIS FOR CLUTCH SIZE

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ABSTRACT
Functional enrichment analysis for transcription factors (TF) was carried on a list of non-additively expressed genes obtained from transcriptome analysis of pre-hierarchical follicles of two purebred (Rhode Island Red and White Leghorn) and two reciprocal crossbred (Rhode Island Red × White Leghorn and White leghorn × Rhode Island Red) laying hens. The crossbred hens exhibited heterosis for clutch size Results showed that CCAAT/enhancer binding proteins were significantly enriched in the pre-hierarchical follicles of the hybrid hens. These TF may play critical roles in the progressive growth of the follicles and perhaps, egg laying efficiency in the hybrid hens.
Keywords: Transcription factors, heterosis, hens, clutch

INTRODUCTION
Crossbreeding and selection have been the bedrock for continuous evolution of new strains and breeds of highly prolific laying hens, and heterosis is one of the two major benefits of crossbreeding. The other benefit being breed complementarity. In laying hens, heterosis is the improved resilience and performance in egg laying traits in the crossbred individuals over their parental purebred parents (Bell *et al*., 2002). The search for molecular mechanism driving heterosis has intensified in the recent past, partly because the phenomenon of heterosis is extensively deployed in breeding of agriculturally important species and partly because it is an interesting scientific puzzle on its own right.
The phenomenon of heterosis has been a subject of genetic investigations since the era of Darwin and it has been linked to non-additive modes of gene action, and is therefore not transmitted to next generation. The hypotheses used to explain its occurrence include dominance, over-dominance, under-dominance and epistasis. From gene expression point of view however, molecular factors driving it are still elusive. Transcriptional regulation has been a key event that modulates gene expression and has been inferred to contribute to hybrid vigour (Botet and Keurentjes, 2020).
Transcription factors (TF) are responsible for the regulation of transcription and have been linked to a variety of molecular and cellular functions including cell proliferation, enhanced gene expression and gene silencing. However, there is limited information in the literature concerning TF related to heterosis in laying hens. This study therefore aimed to identify important TF enriched in genes with non-additive mode of expression in the follicles of hybrid hens via in silico analysis.

MATERIALS AND METHODS
Three laying hens each from the purebred (Rhode Island Red and White Leghorn) and crossbred (Rhode Island Red × White Leghorn and White leghorn × Rhode Island Red) populations were selected such that their clutch traits corresponded to the average of their respective populations. Details of the crossing of the parental pure lines and management of the experimental groups were reported in (Isa *et al*., 2020).
Selected hens were dissected by ventral midline incision and the intact reproductive tract of the hens were collected, weighed and separated into ovary (and follicles) and oviduct. Pre-selected follicles were collected from each hen and then frozen in liquid nitrogen and later stored at -80° C until RNA extraction.
Total RNA was purified and quantified using standard protocol and mRNA libraries were constructed and sequenced in an Illumina platform that generates 150 bp paired-end reads. Adaptor-polluted reads, low quality reads and uncharacterized bases were filtered from the raw reads. Clean reads were mapped to the reference genome using HISAT2 (v. 2.0.5) while transcript assembly was achieved

using StringTie2 (v.1.3.2d). Expression level of transcripts in fragments per kilobase of million mapped reads (FPKM) was estimated using HTSEq (v 0.14.0).

Transcripts expression was pairwise compared between the groups; R vs. W, R vs. RW, R vs. WR, WR vs. R and W vs. WR. Reciprocal hybrids were also compared with a synthesized mid-parent transcript expression values, which were calculated as the average of normalized transcript count from combinations of pure lines (1/2(R+W)). DEGseq package (v1. 18.0; Wang *et al.* 2010) was used for differential gene expression analysis. Non-additive modes of gene expression were delineated into dominance, over-dominance and underdominance (Swanson-Wagner *et al.*, 2006). Genes with non-additive expression common in the two hybrids were obtained. To gain insight into the functions of mRNAs with potential influence on heterosis for clutch size, list of non-additively expressed genes was used for reactome pathway and TF enrichment analyses in g. Profoler using default parameters (Raudvere *et al*., 2019).

RESULTS AND DISCUSSION

Heterosis for clutch size and other egg production traits in the experimental populations used in this study was reported in detail elsewhere (Isa *et al*., 2020). Briefly, heterosis in clutch size was 30% and 45% in the Rhode Island Red × White Leghorn and White leghorn × Rhode Island Red crossbred, respectively. Filtering of the RNA-seq reads yielded clean bases above 1.4×10^{10} and over 90% of raw reads had Phred score above 30 in all the samples, which were retained for downstream analyses (Figure 1).

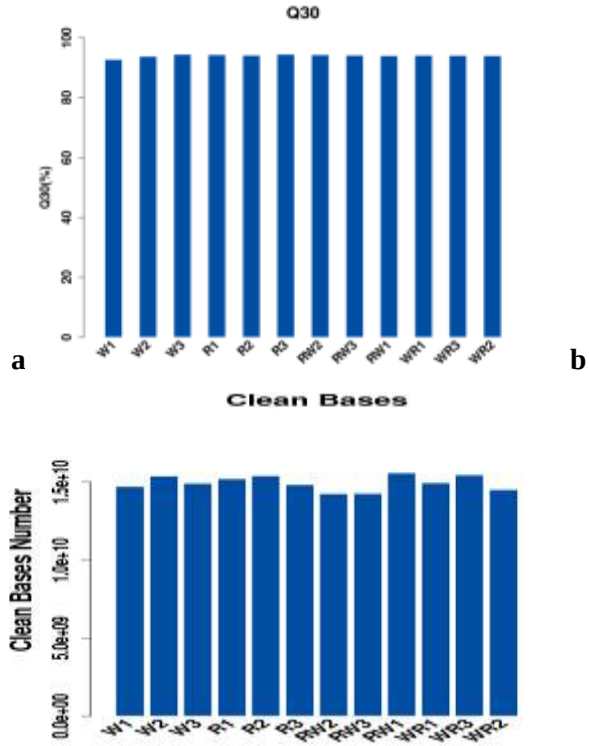


Figure 1: RNA reads quality control statistics for samples (a) proportions of clean reads with Phred score of ≥30 (b) number of clean bases

Result of the differentially expressed genes between all the pairwise comparisons between groups is presented in a heat map (Figure 2).

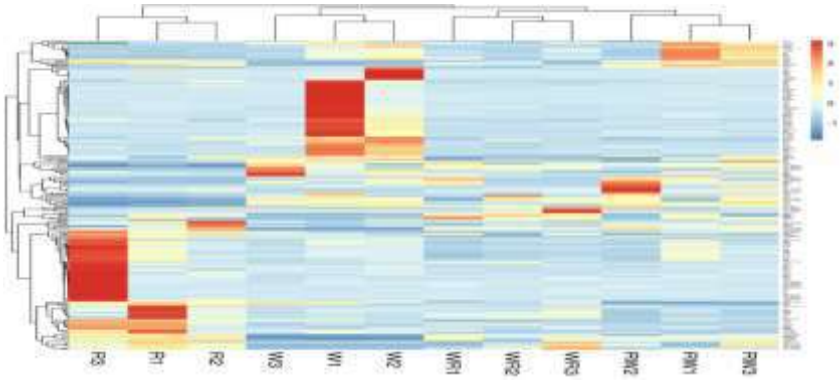
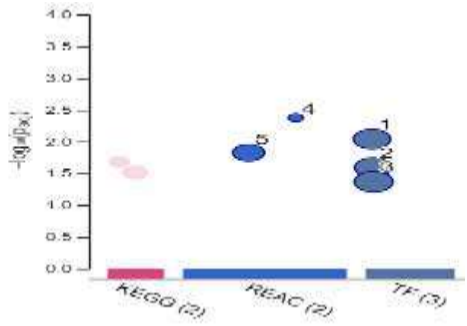


Figure 2: Heatmap for genes with differential expression between groups

Further delineation of gene expression revealed a total of 50 genes that exhibited dominance modes of expression in both hybrid populations. Genes that exhibited any form of non-additive expression in only one hybrid were discarded and not used in the enrichment analysis. Earlier investigations have reported the central role of dominance in heterosis for abdominal fat (Mai *et al*., 2019) and resilience to heat stress in the leukocytes of beef cattle crosses (Zhang *et al*., 2020). Similarly, dominance was documented as the main mode of inheritance pattern in hybrid chickens generated from layer and broiler parental breeds (Gu *et al*., 2019).

Functional enrichment analysis of the genes that exhibited non-additive expression common in the two crossbred groups carried out in g.Profiler revealed that the genes were enriched in CCAAT/enhancer binding proteins (C/EBP [TF-M00190], C/EBPalpha [TF-M00116] and C/EBPbeta [TF-M07315]) TF. Additionally, two reactome pathways (RUNX1 regulates transcription of genes involved in the differentiation of keratinocytes [REAC:R-GGA-8939242] and innate immune system [REAC:R-GGA-168249]) were significantly enriched in genes with non-additive expression (Figure 3).



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ID	Source	Term ID	Term Name	p_{adj} (query_1)
1	TF	TF:M00116	Factor: C/EBPalpha; motif: NNATTRCNNAANNN	9.091×10^{-3}
2	TF	TF:M07315	Factor: C/EBPbeta; motif: NNTTKCNNMAN	2.560×10^{-2}
3	TF	TF:M00190	Factor: C/EBP; motif: NNATTGCNNAANNN	4.277×10^{-2}
4	REAC	REAC:R-GGA-8939242	RUNX1 regulates transcription of genes involved in differentiation of keratinocytes	4.194×10^{-3}
5	REAC	REAC:R-GGA-168249	Innate Immune System	1.488×10^{-2}

Figure 3: Reactome pathway and transcription factor enrichment analysis (a) Manhattan plot showing enriched reactome pathways and transcription factors (b) Details of the enriched reactome pathways and transcription factors

The two C/EBPs TF enriched in genes that exhibited non-additive expression in the pre-hierarchical follicles are members of the Leucine zipper proteins that have been linked to a variety of cellular phenotypes and processes. It has been established that C/EBPα inhibits the proliferation of specific cell types by arresting two cyclin-dependent kinases (Wang *et al*. 2001). In laying hens, progressive development of the pre-selected follicles has been linked to increased efficiency in laying (Johnson and Lee, 2016; Shen *et al*., 2017) where growth is highly regulated.

On the other hand, C/EBPβ jointly with other molecular factors was reported to play a central role in ovulation. In pre-ovulatory follicles, C/EBPβ expression is promoted by luteinizing hormone and disruption in C/EBPβ has been shown to affect gonadotropin biosynthesis and reproductive defects (Fan *et al*., 2011; Zhen *et al*., 2014). Further, C/EBPβ was reported to regulate 3β-hydroxysteroid dehydrogenase, an important enzyme that catalyzes steroid hormone biosynthesis (Li *et al*., 2013). Taken together, these results suggest that C/EBPs may play a crucial role of transcriptional regulation in the pre-hierarchical follicles of hybrid hens that lay larger clutches.

CONCLUSION

By these results, C/EBPs (α and β) have positioned themselves as likely drivers of heterosis for clutch size through their ubiquitous involvement in a variety of cellular processes including steroid hormone biosynthesis and highly regulated growth.

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