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The impact of endogenous Avian Leukosis Viruses (ALVE) on production traits in elite layer lines

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ABSTRACT Avian Leukosis Virus subgroup E (ALVE) integrations are endogenous retroviral elements found in the chicken genome. The presence of ALVE has been reported to have negative impacts on multiple traits, including egg production and body weight. The recent development of rapid, inexpensive and specific ALVE detection methods has facilitated their characterization in elite commercial egg production lines across multiple generations. The presence of 20 ALVE was examined in 8 elite lines, from 3 different breeds. Seventeen of these ALVE (85%) were informative and found to be segregating in at least one of the lines. To test for an association between specific ALVE inserts and traits, a large genotype by phenotype study was undertaken. Genotypes were obtained for 500 to 1500 males per line, and the phenotypes used were sire-daughter averages. Phenotype data were analyzed by line with a linear model that included the effects of generation, ALVE genotype and their interaction. If

genotype effect was significant, the number of ALVE copies was fitted as a regression to estimate additive ALVE gene substitution effect. Significant associations between the presence of specific ALVE inserts and 18 commercially relevant performance and egg quality traits, including egg production, egg weight and albumen height, were observed. When an ALVE was segregating in more than one line, these associations did not always have the same impact (negative, positive or none) in each line. It is hypothesized that the presence of ALVE in the chicken genome may influence production traits by 3 mechanisms: viral protein production may modulate the immune system and impact overall production performance (virus effect); insertional mutagenesis caused by viral integration may cause direct gene alterations or affect gene regulation (gene effect); or the integration site may be within or adjacent to a quantitative trait region which impacts a performance trait (linkage disequilibrium, marker effect).

Key words: ALVE, *ev* genes, trait associations, endogenous retrovirus, production traits

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INTRODUCTION

Endogenous retroviruses (ERVs) were first identified in chicken (*Gallus gallus*) and have since been found ubiquitously in other vertebrate genomes (Weiss, 1967). These transposable elements are the remnants of historical retroviral integrations which may continue to elicit genomic and immunological stress on the host (Llorens et al., 2011; Magiorkinis et al., 2012; Payne and Nair, 2012; Stoye, 2012; Hurst and Magiorkinis, 2015).

ERVs comprise approximately 3% of the avian genome, although the majority of ERVs have been degraded or epigenetically silenced (Stoye, 2001; Yu et al., 2008; Mason et al., 2016; Hu et al., 2017; Kapusta and Suh, 2017), so ancient relics may be difficult to detect, thus the content may be even higher.

In chickens, the only retrovirus with recurrent exogenous and endogenous activity is Avian Leukosis Virus (ALV; Borysenko et al., 2008; Payne and Nair, 2012). ALV is an alpharetrovirus known to infect all Galliform birds (Payne et al., 1991). The endogenous subgroup E (ALVE; also known as *ev* genes) is endemic only to chicken and the red junglefowl progenitor (Frisby et al., 1979; Venugopal, 1999). Many ALVE remain at least partially functional due to their recent integration into the chicken genome (Benkel, 1998; Weiss, 2006). Fully

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replication competent proviruses retain the ability to shed viral particles, facilitating horizontal ALVE transmission and persistent host viremia (Bacon et al., 1988; Gavora et al., 1995; Chang et al., 2006). Viremia has been shown to significantly delay and reduce antibody production to exogenous ALV (Smith et al., 1990, 1991; Gavora et al., 1995; Yu et al., 2008).

Not all ALVE are structurally intact, but expression of individual proteins encoded by the viral *gag*, *pol* and *env* genes can still elicit a significant effect on the host (Astrin and Robinson, 1979; Robinson et al., 1981). Production of *gag* polyproteins has been shown to induce tolerance to novel ALV infections, resulting in a delayed immune response and higher incidence of lymphoid tumors (Astrin et al., 1979; Crittenden et al., 1984). Contrastingly, *env* glycoproteins can also confer resistance to novel infections through receptor interference (Robinson et al., 1981; Lamont et al., 1992; Yu et al., 2008). Whilst the ALVE copy number in individual birds is low (typically fewer than 10, even in noncommercial flocks (Mason et al., 2020a,b) the variable expression and integrity of each element can lead to complex interactions and effects, including inter-element regulation (Robinson et al., 1981; Crittenden et al., 1984; Smith et al., 1990; Kuhnlein et al., 1993).

ALVE are known to be present in commercial chicken genomes although ALVE-free lines have been generated (Astrin et al., 1979; Zhang et al., 2008). The presence of ALVE in the chicken genome has been shown to impact numerous traits including sexual maturity, egg weight, body weight, and egg production in layer breeds (Kuhnlein et al., 1989; Gavora et al., 1991; Iraqi et al., 1994). Some specific ALVE are closely associated with desirable commercial traits. ALVE21 is physically linked with the slow feathering phenotype used for determining the sex of day-old chicks (Bacon et al., 1988; Levin and Smith, 1990; Iraqi and Smith, 1995; Elferink et al., 2008; Takenouchi et al., 2018). ALVE-TYR is an insertion within the *Tyrosinase* gene, responsible for the recessive white mutation (Chang et al., 2006) known to have a negative impact on growth rate (Fox and Smyth, 1985; Pardue et al., 1985).

ALVE were originally detected by genomic digestion with restriction enzymes, followed by Rous Sarcoma Viral probe hybridization (RFLP/Southern blot; Astrin, 1978). Early work identified 23 ALVE in the White Leghorn (WL) breed, with an additional 20 to 25 in other chicken breeds including commercial broilers (Sabour et al., 1992; Gorbovitskaia et al., 1993; Benkel, 1998; Hunt et al., 2008). This RFLP/Southern blot detection approach was time consuming, expensive and not suitable for rapid testing of large numbers of samples. Subsequently, more rapid PCR-derivative methods with gel-based detection were developed for identification of many of the ALVE (Benkel et al., 1992; Benkel and Smith, 1993; Benkel, 1998; Smith and Benkel, 2009). Again, however, these approaches were not scalable to entire flocks, and incomplete ALVE identification further limited eradication from commercial lines. The availability of rapid and low-cost sequencing

methodologies has resulted in whole genome sequence information being obtained from multiple commercial and experimental chicken lines (Kranis et al., 2013). ALVE integrations can therefore be identified bioinformatically, facilitating the development of integration-specific, rapid PCR assays with fluorophore-labeled primers to enable allele-aware automation at scale.

Recently, twenty ALVEs were identified across eight elite layer lines from whole genome sequencing (WGS) data using obsERVer, a new bioinformatic pipeline developed for identifying retroviral integrations (Mason et al., 2020b). Fifteen (75%) of these ALVE inserts had been previously reported (Gavora et al., 1991; Grunder et al., 1995; Benkel, 1998; Rutherford et al., 2016). For each of these 20 ALVE inserts, a highly specific, rapid and inexpensive PCR-based assay with detection by allele-specific fluorescence was developed (Mason et al., 2020b). Each assay was designed to identify the presence and absence of the ALVE insert, thus both homozygotes and the heterozygote genotypes were definitively identified. Seventeen (85%) of the ALVE segregated within at least 1 elite line. These assays were then used to obtain ALVE genotype information for males from 8 elite lines and multiple generations (10-19 generations), each with daughter performance data, to identify associations between ALVE integrations and 18 commercially relevant layer traits.

MATERIALS AND METHODS

Genetic Material and Trait Information

DNA and performance trait information were obtained from 8 elite layer genetic lines, accumulated over 10 to 19 generations per line. Three different breeds were used; 5 White Leghorn (WL1-5) lines, 2 White Plymouth Rock (WPR1-2) lines, and one Rhode Island Red (RIR1) line. Genotypes were obtained from males, and averaged daughter phenotypes for each sire was used as the phenotypic value. Not all traits were measured for all generations. The number of genotyped males ranged from 500 to 1500 per line.

The association study assessed 18 traits which are under current selection. There were three egg production traits: production (PD; the percent egg production over the life of each hen), egg number (EN; total number of eggs produced per hen), and sexual maturity (SM; age of first egg for each hen). Nine traits were specifically related to egg quality. Early egg weight (E3) and shell color (C3) were measured on the first 3 eggs laid. The other seven egg quality traits were average values of eggs laid between 26 and 80 weeks of age and included egg weight (EW), yolk weight (YW), shell color (CO), albumen height (AH), breaking strength (BS), puncture score (PS), and dynamic stiffness (Kdyn). Color was measured in an index, which combines the L*, a*, and b* parameters of the Minolta Chromameter system (Tokyo, Japan). EW was measured in grams with a precision of at least 0.1 g, AH was measured in mm, BS in gF, and PS in g at 1.0 mm/s. Kdyn is a quantitative

measure of resistance over the entire egg surface (N/cm) obtained by an acoustic test, which also detects micro-cracks in the shell. A more detailed description of this trait can be found in [Arango et al. \(2016\)](#). Finally, three mortality traits were included: mortality during the grow period (Gmort; hatch through 15 weeks), lay period (Lmort; 16 weeks through 70-90 weeks of age) and progeny mortality following Marek's Disease virus challenge (MDmort; [Fulton et al., 2013](#)). Body weight information collected at 18 weeks of age (BW18) and at 32 weeks of age (BW32) was also included, as was the residual feed intake (**RF**), measured as the residuals from a regression model for total feed intake after adjusting for metabolic body weight ($BW^{0.75}$) and egg mass measured during the two week feed test period.

Previously characterized quantitative trait regions (**QTR**; ChickenQTLdb; www.animalgenome.org/QTLdb/chicken/; accessed 06/29/2020; [Hu et al., 2019](#)) associated with the traits outlined above, were compared to chromosomal locations (within a 4 Mbp window) of the 20 ALVE integrations previously identified in these elite layer lines ([Mason et al., 2020b](#)). ChickenQTLdb trait information has been identified from multiple breeds and likely include variable, but reliable, phenotypic measurement methodologies. However, indications of existing genomic locations associated with traits may represent trait association by linkage alone, although prior incomplete ALVE annotation should not be dismissed.

Genotyping

The development of novel, genotype-discriminating, detection assays for the 20 ALVE inserts present within the lines utilized for this study has been previously described ([Mason et al. 2020b](#)). Briefly, these assays use Kompetitive Allele-Specific PCR (KASP;

LGC, UK) which allows visualization of alleles through end-point read allele-specific fluorescence. The presence of each ALVE within a line was first determined by genotyping one generation of males. Subsequently, ALVE assays were genotyped on DNA from only those lines in which they were segregating. Genotype data was generated for 17 ALVE inserts and analyzed using Kraken software (LGC, Middlesex, UK). The 3 remaining ALVE assays (ALVE9, ALVE21, and ALVE-TYR) showed genotype fixation (either absence or presence within each line) and thus trait associations could not be examined.

ALVE-Trait Association Tests

All genotype classes with less than 5 segregating observations were removed prior to the analysis. The association analysis was performed using the lm (Fitting Linear Model) procedure in R ([R Core Team, 2020](#)). In the first step, a model with fixed effects of generation, ALVE genotype and their interaction was fitted for each trait and line separately.

$$y_{ijk} = \mu + \text{generation}_i + \beta \text{ALVE}_j + \text{generation.ALVE} + e_{ijk}.$$

where

y_{ijk} is the trait phenotype for kth hen from i-th generation with ALVE genotype j,
 β is the regression coefficient,
 generation.ALVE is the interaction term,
 e_{ijk} is the residual.

For traits with a significant ($P < 0.05$) main effect of ALVE and a nonsignificant ($P > 0.1$) ALVE by generation interaction, the interaction was dropped from the

Table 1. Segregation of ALVE in elite layer lines under study.

ALVE	WL1	WL2	WL3	WL4	WL5	WPR1	WPR2	RIR1	Status ¹
ALVE1			+	+					Full
ALVE3		+	+	+				+	Full, lacks RT
ALVE15	+	+			+				solo L
ALVEB5						+	+	+	Full
ALVE-NSAC1						+	+	+	P,E,L
ALVE-NSAC3						+	+		Unk
ALVE-NSAC7						+	+		Full
ALVE-ros001								+	Full
ALVE-ros002								+	Unk
ALVE-ros003								+	Full
ALVE-ros004							+	+	Full
ALVE-ros005								+	solo L
ALVE-ros006								+	Unk
ALVE-ros007								+	Env, L
ALVE-ros008				+					Full
ALVE-ros009							+		Unk
ALVE-ros010								+	Unk

Abbreviations: E, envelope; L, 3' long terminal repeat; P, polymerase; RT, reverse transcriptase; Unk, gene status unknown.

¹From [Mason et al., 2020b](#).

model and number of ALVE copies was fitted as a regression in order to estimate additive ALVE effect. The remaining ALVE by trait combinations were removed from consideration.

RESULTS AND DISCUSSION

A total of 20 ALVE were tested in the elite lines utilized in this study (Mason et al. 2020b). Table 1 shows the 17 ALVE that were found to be segregating within at least one line. Table 1 also indicates status or completeness of each ALVE from Mason et al. (2020b), where known. The number of ALVE segregating within each line was variable and ranged from 1 to 3 in the WL lines, 5 to 6 in the WPR lines and 11 in the RIR line (Table 1), and many ALVE are breed-specific.

ALVE Association With Layer Performance Traits

The presence of each segregating ALVE within the eight elite layer lines was tested for association with 18 performance traits relevant to commercial egg production. Across all lines, there were a total of 30 segregating ALVE (from 17 individual loci segregating within at least one line), thus providing a total of 540 association tests (30×18 traits), summarized in Table 2. Fifteen of the 17 ALVE exhibited significant associations with at least one trait in at least one line, with only the low frequency ALVE-ros001 and ALVE-ros002 (both in RIR1) excluded. We observed more significant associations than expected under a random Gaussian distribution (11 at $P < 0.001$; 32 at $P < 0.01$; 56 at $P < 0.05$) suggesting that within these elite layer populations there were indeed associations between the presence/absence of some ALVE inserts and measured performance traits. Furthermore, despite the literature focus on the detrimental effects of ALVE on productivity (Kuhnlein et al., 1989; Gavora et al., 1991; Iraqi et al., 1994) 37.5% of significant ($P < 0.05$) associations (21/56) were positive, that is, the presence of the ALVE showed a positive impact on selection phenotypes within the context of the breeding program.

The analyses revealed multiple examples where individual ALVE elicited different impacts on the same trait in different lines. Traits significantly affected by the presence of an ALVE in one line may be unaffected in different lines with the same ALVE, such as ALVE-NSAC1 with BW in WPR2 but not in WPR1 or RIR1. Furthermore, the presence of the same ALVE integration may have a positive effect on a trait in one line, but a negative effect in another, seen with AH for both ALVE15 (WL2 vs. WL5) and ALVE-ros004 (WPR2 vs. RIR1). Other integrations appear to have diverse associations in different lines. ALVEB5 was present in WPR1, WPR2 and RIR1, but significantly impacted the very different traits of C3 in WPR1, BS in WPR2, and E3 in RIR1 within the lines. In addition, within a single line, one ALVE may associate significantly with multiple

traits. Within WL2, ALVE3 impacts AH, PS, and %MDmort, within WL3 it impacts PD, AH, PS and EW, whereas within WL4 it impacts EN, C3, AH and E3, with only AH consistently modulated (negatively) between the 3 lines. These incongruent or variable associations may reflect complex ALVE interactions in each line, including with ALVEs not segregating within those lines. ALVE9, known to produce envelope glycoproteins like ALVE3 (Robinson et al., 1981) was fixed within WL3 and found in no other WL lines, and similarly for the structurally intact ALVE21 in WL4. Notably, 6 ALVE exhibited only detrimental trait associations, all within the WPR and RIR: ALVE-NSAC1 (across both breeds), ALVE-NSAC3, ALVE-NSAC7, ALVE-ros005, ALVE-ros007, and ALVE-ros009.

The dataset utilized for this study is unique as it includes three breeds (WL, WPR, RIR) with 2 represented by multiple lines (WL1-5, WPR1-2) with the same traits measured across all birds, providing the opportunity to independently assess trait associations in more than 1 line and breed. ALVE integration structural integrity was known for 12 of the 17 ALVE studied here (Table 1; Mason et al., 2020b). Structural integrity does not appear to be a prerequisite of significant trait associations, with 27/56 (48.2%) associated with ALVEs known to be incomplete. ALVE3 has 11 significant associations in WLs (but none in RIR1), is intronic within *HCK*, (*HCK* proto-oncogene), and is known to produce *gag* and *env* glycoproteins, but has no *reverse transcriptase*. Interestingly, the 2 solo LTRs in this dataset (lacking any retroviral genes), ALVE15 and ALVE-ros005, both exhibit significant trait associations. ALVE15 shows 1 to 5 significant trait interactions in each of the 3 WL lines in which it is found (Table 2). The presence of ALVE15 within the final intron of *GRIK2* (glutamate ionotropic receptor kainate type subunit 2) might disrupt gene transcription (as is seen with ALVE-TYR) and the final exon is known to regulate channel dynamics in related receptors (Maki et al., 2012). Solo LTRs may also provide alternative promoter activity, which could explain the highly significant impact of the intergenic ALVE-ros005 on C3. Interestingly, an EAV LTR was previously shown to cause blue eggshell color by providing an alternative promoter sequence (Wang et al., 2013; Wragg et al., 2013).

Likelihood of the Direct Impact of ALVEs on Performance Traits

There are multiple mechanisms that may explain the relationships between the presence of ALVE in the chicken genome and various production traits. *First*, those ALVE that are fully competent (e.g., ALVE1) could produce viral particles, with the potential to cause negative health impacts, thus indirectly impact production traits. Variation in impact between different lines could be due to inhibition of viral protein production in some genetic backgrounds (including by other ALVE; Robinson et al., 1981), or resistance to retroviral (re)

entry into cells (Yu et al., 2008). However, the elite lines reported herein are intensively selected lines, with selection including the elimination of all birds showing positive ELISA detection of the ALVE *gag* p27 protein, likely selecting against total viral protein production. Indeed, the *gag* ORF appears disrupted across p10/p27 in 6 of the 8 ALVE studied here which retain the *gag* domain (Mason et al., 2020b). Different genetic backgrounds could have more or less of this ‘inhibition’ and could lead to different results seen for effect vs. no effect on performance traits, although specific associations with individual, often inter-correlated traits, might suggest an alternative mechanism than broad physiological effects. *Second*, the genomic ALVE integration site could interrupt genes or interfere with gene regulation (direct, causative effect). ALVE-TYR, found to be fixed in WPRs and the Rhode Island White breed (Mason et al., 2020b), is an example of an ALVE that impacts a specific gene, as the presence of this insert within the *TYR* final intron results in lack of pigmentation and the consequent recessive white phenotype (Chang et al., 2006). ALVE1, ALVE3 and ALVE15 studied here are all intronic integrations, although there is no evidence of ALVE-mediated transcript dysregulation. *Third*, an ALVE insert could be located nearby to a gene or quantitative trait region (QTR) which has an impact on a trait. This would be considered a marker effect and could be different between lines due to linkage disequilibrium (LD). LD may be the most likely explanation for why the same ALVE associates with distinct traits in different lines (e.g., ALVEB5), and why the magnitude of the associated effect may disappear or even reverse between lines (e.g., ALVE15 and ALVE-ros004 with AH; ALVE3 between WLs and RIR).

Examination of the Animal QTLdb (www.animalgenome.org accessed 06/29/2020; Hu et al., 2019) enabled comparison of ALVE position relative to previously identified QTR related to the traits of interest. Previously annotated QTR were identified using data from multiple breeds, and the phenotypic measurement approaches likely differ between studies. QTR identified within 2 Mbp up- and downstream of each ALVE were best-matched with the measured Hy-Line traits from this study (Table 3) and considered in the context of the observed significant associations identified in this study. There are common QTR (9/17, 53%) between those reported in the Animal QTLdb and those found in the studied elite lines in this paper. ALVE3 is identified as impacting various egg related traits in both the QTL data base and the Hy-Line elite lines, consistent with the multiple reports of detrimental effects in the literature (Gavora et al., 1991), although this study also identified a positive impact on egg production in WL3. ALVE15 impacts shell color, ALVE-NSAC1 impacts BW and ALVE-NSAC3 impacts RF in both data sources. ALVE-ros009 impacts YW and ALVE-ros010 impacts EN related to PD in both data sets. Inconsistencies between the two data sources could be due to lack of segregation or detection of ALVE (particularly those that are rare) in the studies included within the Animal

Table 3. Quantitative Trait Regions (QTR) nearby to ALVE segregating in Hy-Line lines (from ChickenQTLdb; www.animalgenome.org/QTLdb/chicken/), and the phenotypes impacted.

ALVE	Location	QTR in Animal db	Traits in Hy-line
ALVE1	1:65,993,542	BW, EW, ST	C3, kDyn, Gmort%
ALVE3	20:10,309,347	BW, CO, EN, EW, SM	EN, PD, E3, EW, C3, AH, PS, Mdmort%
ALVE15	3:70,384,294	CO, SS	CO, C3, AH, PS, Lmort%, BW18
ALVEB5	1:10,637,460	none	E3, C3, BS
ALVE-NSAC1	2:120,868,843	BW, CO	BW, RF, Mdmort%
ALVE-NSAC3	3:53,639,776	BW, RF	EN, RF, SM, Mdmort%
ALVE-NSAC7	9:11,714,130	EN	BW18, EW
ALVE-ros001	1:101,668,931	none	none
ALVE-ros002	1:158,775,708	BW	none
ALVE-ros003	1:163,248,553	BW	SM
ALVE-ros004	2:124,432,997	none	BW18PD, EW, YW, CO, AH, BS
ALVE-ros005	2:142,480,536	SYFN	C3, SM
ALVE-ros006	3:57,337,987	BW	E3, EW, AH
ALVE-ros007	4:59,843,015	none	BS, YW
ALVE-ros008	4:62,680,158	none	MDmort%
ALVE-ros009	4:71,095,932	BW, SM, SYFN, YW	EW, YW
ALVE-ros010	9:11,871,576	EN	AH, BW18, CO, PD

Relevant phenotypes affected by these same ALVE in Hy-Line genetics are also shown.

Abbreviations: BW, body weight; CO, shell color; EN, egg number; RF, residual feed intake; ST, shell thickness; SM, sexual maturity; SS, shell strength; SYFN, small yellow follicle number; YW, yolk weight.

QTLdb, or impact of background genes on trait expression. Multiple smaller-scale studies previously utilized the WL breed to examine associations between ALVEs and various commercially relevant traits (Kuhnlein et al., 1989; Gavora et al., 1991; Aggrey et al., 1998). Of those reported here, ALVE3 is the most studied (Robinson et al., 1981; Gavora et al., 1991), with results largely consistent with the present study. While some of the ALVE/trait associations presented are the same as have been identified previously, others cannot be assessed completely. One important omission from this study is ALVE6, an element located within repetitive sequence near the chromosome 1 p-arm telomere (inhibiting development of a high-throughput assay; Mason et al., 2020c) known to modulate ALVE activity and affect host physiology (Robinson et al., 1981; Levin & Smith, 1990). Furthermore, the fixed ALVE in these lines (ALVE9, ALVE21, and ALVE-TYR) also have previously identified modulatory effects, require segregation to be studied by association analysis.

CONCLUSIONS

Previous reports have indicated impact of ALVE presence on multiple traits including 8% to 9% reduction in annual egg production, 2.2 g decrease in egg weight and 0.003 reduction in egg specific gravity (and indicator of shell strength; Gavora et al., 1991), yet in the intensively selected elite egg production lines at Hy-Line

International, segregating ALVE persist. It would be expected that any genetic variations (including ALVE inserts) that have a negative impact on production traits would have been eliminated. The data and analyses presented here show that segregation of ALVE persists within commercial egg production lines and that their presence can have an impact on production traits. Furthermore, this impact is not necessarily negative, and can be line dependent. This may be partially related to the multiple trait selection emphases with different selection pressures being placed on different traits. Alternatively, negative effects may be compensated by positive effects on other traits, resulting in a balanced equilibrium within lines as proposed by Iraqi et al. (1991). Whilst multiple ALVE exhibit varying incongruent associations with similar traits consistent with linkage disequilibrium, the identification of six ALVE with exclusively detrimental effects, five of which are associated with QTRs for relevant traits, may provide new markers to benefit the efficiency of existing breeding programs.

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DISCLOSURES

The authors declare there is conflict of interest.

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