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B.K. Podisi, S.A. Knott, I.C. Dunn, D.W. Burt and P.M. Hocking

B.K Podisi - Department of Agricultural Research, P/Bag 0033, Gaborone, Botswana

S.A.Knott - Institute of Evolutionary Biology, University of Edinburgh, King's Buildings, West Mains Road, Edinburgh, EH9 3JT, Scotland, UK

I.C.Dunn, D.W.Burt and P.M Hocking - The Roslin Institute and Royal (Dick) School of Veterinary Studies, University of Edinburgh, Easter Bush, Midlothian EH25 9RG

Abstract

1. An F(2) cross of a broiler male line and a White Leghorn layer line was used to identify quantitative trait loci (QTL) for bone density at the onset of lay and at the end of the laying period. A total of 686 measures of humeral bone density were available for analysis. 2. There was no evidence for epistasis. 3. Genome-wide significant QTL for bone density at the onset of lay were identified on chromosomes 1 (311 cM) and 8 (2 cM) and on chromosomes 1 (311 cM), 3 (57 cM) and 8 (2 cM) with a covariate for the number of yellow follicles (a proxy for the concentration of circulating oestrogen). 4. Evidence for only 4 chromosome-wide suggestive QTL were detected at the end of lay (72 weeks). 5. Analysis of the combined data confirmed two genome-wide suggestive QTL on chromosome 1 (137 and 266 cM) and on chromosomes 8 (2 cM) and 9 (10 cM) in analyses with or without the covariate. 6. Positive QTL alleles came from the broiler line with the exception of 2 suggestive QTL at the onset of lay on chromosomes 3 and 5 in an analysis with the covariate. 7. In general, QTL acted additively, except that dominant effects were identified for three suggestive QTL at the onset of lay on chromosomes 3 (57 and 187 cM) and 5 (9 cM). 8. The significant QTL in this study were at similar locations to QTL identified in a range of crosses in other publications, suggesting that they are prime candidates for the search for genes and mutations that could be used as selection criteria to improve bone strength and decrease fractures in commercial laying hens.

Introduction

Bone mineral density (BMD) is a common measure of susceptibility to osteoporotic fractures in both humans and other species, including chickens (Whitehead and Fleming, 2000; Johnson et al., 2009). Bone fragility in humans is due to the decline in oestrogen after menopause but in chickens the decline in the structural integrity of the bones is caused by mobilisation of cortical bone for egg laying (Rubin et al., 2007). Osteoporosis is not only an animal welfare issue but affects productivity and leads to processing losses in the industry (Silversides et al., 2006). It is the underlying cause of bone fractures of the humerus and keel and is a consequence of the loss of structural bone caused by the demands of high rates of egg shell formation in modern layers (Whitehead and Fleming, 2000; Webster, 2004). Exercise and good nutrition can lead to stronger bones and reduced fractures and the beneficial effects can be complemented with genetic selection to improve bone strength and resistance to osteoporosis (Fleming et al., 2006).

Osteoporosis is evident in laying hens from 35—45 weeks of age onwards (Cransberg et al., 2001) and is the result of prolonged exposure to high levels of oestrogen and the demands of egg laying (Beck and Hansen, 2004). As hens approach sexual maturity the reproductive system becomes functional, oestrogen concentration increases and stays elevated during egg production. The source of the oestrogen is principally the smaller white and the early yolky hierarchical follicles (Armstrong, 1984; Robinson and Etches, 1986). Because the number of follicles varied widely in the cross in the present experiment the number of yellow follicles was measured as a potential factor influencing BMD.

Oestrogen promotes calcium absorption and the formation of the specialised medullary storage bone (Dacke et al., 1993). A decline in oestrogen receptor number with age may contribute to a reduction in the efficiency of these adaptations for the demands of egg shell formation (Beck and Hansen, 2004). The partitioning of key metabolites such as calcium for bone deposition, egg production and other homeostatic functions could be controlled by a number of genetic factors or genes. Osteoporosis in humans is also an age-related condition and is influenced not only by genetic factors but also by environmental, gene-gene and gene-environmental interactions (Johnson et al., 2009). It is therefore possible that epistasis may be involved in the biological process of osteoporosis in laying hens.

Besides nutritional and environmental interventions, osteoporosis can be reduced through selective breeding (Bishop et al., 2000; Fleming et al., 2006). A selection index (Bone Index) has successfully been used to select against osteoporosis in laying chickens (Bishop et al., 2000). A QTL for bone index was reported on chromosome 1 at position 370cM in an F_2 population produced from two White Leghorn lines divergently selected on the basis of the bone index (Dunn et al., 2007). Bone mineral density (BMD) is a traditional measure of bone strength (Hans and Krieg, 2008) and identification of QTL for BMD could assist breeding efforts to address osteoporosis. Suggestive QTL for bone mineral density have been detected in an F_2 broiler (Cobb male line) x layer (White Leghorn female) population (Schreiweis et al., 2005). Several QTL for BMD were detected and potential gene candidates were proposed based on a QTL in two F_2 populations from the offspring of a male broiler line crossed with White Leghorn and Fayoumi (Zhou et al., 2007). However, none of these studies focused specifically at the critical period when birds reach sexual maturity. This stage is important for initiating key physiological processes that lead

to egg production, which in turn affect bone density. High BMD is important at the onset of lay because such birds have higher reserves of calcium to support subsequent egg production. These reserves are not replaced after sexual maturity because the deposition of calcium in structural bone is prevented by the oestrogen-dependent switch to medullary bone.

A study was therefore conducted to identify QTL influencing BMD and to investigate the possible role of epistasis in a F2 broiler-layer cross population.

Materials and Methods

Animals and husbandry

The production of the F1 and F2 generations was described by Sewalem et al. (2002). In the grandparent generation two males and two females from the Ross 308 male line broiler (Aviagen, Newbridge, UK) and from a White Leghorn egg laying line maintained at the Roslin Institute were crossed to create 4 F1 families. Eight males and 32 females of the F1 generation were randomly selected and mated in a balanced mating scheme to produce the F2 population. One female died and was replaced for producing the young flock making a total of 33 full sib families.

A total of more than 1000 female offspring from 20 hatches were initially housed in pens of about 20 birds. At 12 weeks of age the birds were moved to individual cages measuring 40 cm wide x 45cm deep x 80cm high until the end of the experiment. Each pen of about 20 birds was housed in a single block of cages on the same tier. The birds were fed ad libitum on conventional diets for laying hens and exposed to a constant photoperiod of 14 hours per day from hatch to the end of the experiment.

The experiments were conducted after local ethical review under Government approved licences to protect the welfare of the birds at all times.

Observations

Hens were culled within 3 d of laying their first egg (young flock, 11 hatches) or at 72 weeks of age at the completion of the egg laying period (old flock, 9 hatches). The onset of lay was defined as the day of first recorded oviposition. The birds were killed by an overdose of sodium pentobarbitone and the abdominal cavity was opened. The ovary was dissected and the number of yellow follicles was recorded. The right wing was dissected from the carcase and stored at -20C. At a later date the wing was thawed and the humerus dissected from adhering tissue including tendons and ligaments. The bone was radiographed on a lateral plane alongside a calibrated aluminium step-wedge as described by Hocking et al. (2003). The density of the image of the whole bone was then compared with the density of the wedge to determine the bone density defined as the density equivalent of a specified depth (mm) of aluminium using NIH-image analysis software.

Genotyping and linkage map construction

Blood samples were collected for DNA extraction and genotyping by superficial venipuncture of a wing vein at 12 weeks of age. DNA was extracted from the sample using standard methods. A total of 106 microsatellite markers covering 26 autosomal linkage groups and the Z sex chromosome (Table 1) were genotyped in sets of 4 to 10 markers based on the fragment size and dye colour of the PCR product. Fluorescent

microsatellite detection was performed on an Applied Biosystems 3730xl genetic analyser (Applied Biosystems/Hitachi, Applera, USA) and Genemapper Software v3.5 (Applied Biosystems, Applera, USA) was used to estimate fragment sizes.

Data management, preparation and cleaning

The data were from two groups of birds, one that was killed at sexual maturity (young flock) and the other at 72 weeks of age (old flock). The average age of the young flock at first egg was 21 weeks (SD 3 weeks). The old flock was sampled at the end of the usual laying period for layers when bone density is expected to be at its poorest.

All pedigrees, marker genotypes and recorded traits were stored in the resSpecies database (Law and Archibald, 2000). Data were edited to detect and eliminate genotyping errors using RTools (Dr Ricardo Pong-Wong, 2007, personal communication). The map files were created through the CRIMAP program (Green et al., 1990) and marker order was confirmed by comparing it to previously published linkage maps (Hu et al., 2001; Navarro et al., 2005).

Execution of the epistatic QTL analysis on GridQTL

Mapping and significance testing were conducted by the interval mapping method for QTL analysis adapted for epistasis detection in GridQTL (Seaton et al., 2006; Wei et al., 2009) as described earlier (Podisi et al., 2011). Significance thresholds for detection of single QTL with significant marginal effects were determined through

5000 permutations (Churchill and Doerge, 1994) and 1000 bootstraps were used to generate 95% confidence intervals for the QTL positions (Visscher et al., 1996).

F-values greater than those corresponding to the $P \leq 0.05$ and $P \leq 0.01$ experiment-wide threshold values, respectively, were used to identify a significant and highly significant QTL (Kruglyak and Lander, 1995). QTL thatachieved an F ratio exceeding the $P \leq 0.05$ chromosome-wide threshold were considered to be suggestive.

Significance testing for epistatic pairs used F ratio tests for model comparisons in a nested test framework following Wei et al. (2009). The following tests were conducted:

Model 1 (With epistasis) : BMD = μ + Locus A

+ Locus B + Locus A x Locus B + e

Model 2 (No epistasis) : BMD = μ + Locus A

+ Locus B + e

Model 3 (Single locus model) : BMD = μ

+ Locus A + e

Model 4 (Null model) : BMD = μ + e

where μ is the model constant, and e is the random error.

An overall F test termed F_{all} was used to compare Model 1 to Model 4 and QTL pairs that passed the F_{all} criteria were subjected to an interaction test denoted as F_{int} by comparing Model 1 and Model 2. To ensure that the aggregate effect of a pair of loci which involved a marginal-effect QTL explained significantly more of the phenotypic

variance than the marginal QTL alone, an overall test was conducted by comparing Model 1 with Model 3. Genome-wide thresholds were derived in advance based on 1000 replicates.

Model definition

Different models with additive, dominance and parent-of-origin genetic effects with family and pen as fixed effects (hatch was confounded with pen) were evaluated in a preliminary analysis. There was no evidence for a parent-of-origin effect (detected as a difference between the alternative heterozygous genotypes that differ in which allele was inherited from each parent) (Knott et al., 1998), and parent of origin effects were ignored in subsequent analyses. The Z chromosome was analysed with an additive genetic effects model for the detection of QTL with significant marginal effects. The epistasis analysis did not include the Z chromosome.

Family and pen were fitted as fixed effects for both data sets. Data for the combined analysis were pre-corrected for pen nested within hatch after fitting a model that included effects for hatch, pen and family. The statistical model for the combined population included the effects of age at the measurement of BMD. The number of normal yellow follicles (NYF) was used as a covariate in a second round of analyses.

Results

The number of records of BMD available for analysis for the young and old layer bird data sets was 388 and 268, respectively (Table 2) and was substantially lower than the total number housed for both sets of data. A number of images could not be

assigned to a bird and a large proportion of the old flock were not in laying condition (birds with no normal yellow follicles).

The phenotypic correlations between BMD and other traits for the two flocks are presented in Table 3. The estimated phenotypic correlations were generally low and positive except for the negative correlation between BMD and age at first egg (AFE). The highest correlation (0.42) was between body weight at 72 weeks (BW72) and BMD (Table 3). There was no correlation between weight at first egg (WFE) and BMD in the old flock in contrast to the young flock.

There was no evidence for epistasis in any of the analyses (data not shown) and the results for single QTL analyses only are presented. Two significant QTL on chromosomes 1 and 8 respectively and 4 suggestive QTL, one each on chromosomes 1 and 4 and two on chromosome 3 were detected in the young flock (Table 4). A significant QTL segregating on chromosome 8 for BMD explained the highest proportion of the phenotypic variation (4.5%) without fitting a covariate in the model. For older chickens evaluated at 72 weeks of age suggestive QTL were detected on chromosomes 2 and 8. The results of the combined analysis (Table 5) led to the detection of 4 suggestive QTL: two on chromosome 1 and one on chromosome 8 at similar locations as in the young flock and one on chromosome 9.

Fitting NYF as a covariate had relatively little effect on the analyses. In the young flock, a suggestive QTL on chromosome 4 was not detected and an additional suggestive QTL on chromosomes 3 and 5 were identified. The significance of the QTL at 305cM on chromosome 1 and at 57cM on chromosome 3 increased, the latter from suggestive to highly significant whereas the F-statistic for the QTL on chromosome 8

was slightly lower (Table 4). For the combined data set the analysis with the covariate produced similar results to that without (Table 5) and is not presented.

The estimated size of the QTL effects is presented in Tables 4 and 5. The detected QTL individually explained from 1.3 to 5.7 % of the phenotypic variation. Most of the QTL had significant positive and additive QTL effects. However, a locus on chromosome 3 at 57 cM had significant dominance action that had a negative effect on BMD. The total proportion of phenotypic variation explained by the genome-wide significant and suggestive QTL without and with the covariate respectively were, respectively, 19 and 24% for the young flock, 8.7 and 9.9% for the old flock and 6.5 and 6.5% for the combined analysis.

Discussion

Broiler parent stock do not suffer from osteoporosis and bone density is high at the end of lay (unpublished data) making a broiler x layer cross particularly valuable for identifying QTL for bone mineral density. The purpose of combining the data from young and old birds was to increase the power of the experiment and the likelihood of detecting epistatic gene action and, as far as we know, this is the first analysis of epistasis for BMD and related traits. Whereas no epistasis or additive QTL were detected in the combined analysis it is possible that metaanalyses or analyses of larger populations with denser genotyping might identify significant QTL. Alternatively, bone density at 72 weeks of age in this cross may not have deteriorated because of the relatively large body weight, high feed intake and low egg production (representing high storage and intake of calcium, and a low requirement for eggshell

formation, unpublished data) and eliminated statistical evidence in the combined data of QTL detected in the young flock.

Most of the QTL effects in the young flock were positive indicating that the broiler allele contributed to the increase in BMD. The location of the QTL detected in all three analyses at 311 or 305cM on chromosome 1 with or without fitting a covariate respectively is similar to the QTL for humeral breaking strength and bone index that were detected on chromosome 1 at 334cM and 370cM in a cross of a White Leghorn layer line selected for a decrease or an increase in bone strength (Dunn et al., 2007). The location of the QTL on chromosome 1 at 131cM is similar to a reported QTL for femoral BMD at 138cM in a White Leghorn x red jungle fowl cross (Rubin et al., 2007). The proportion of phenotypic variation explained by the QTL on chromosome 3 at 57 cM with a covariate is similar to the 6% reported for a whole-body BMD QTL on chromosome 3 by Rubin et al. (2007). Schreiweis et al. (2005) reported significant QTL for BMD on chromosomes 3, 4 and 27 compared to our study where suggestive QTL on chromosomes 3 and 4 but not 27 were identified. Other QTL detected on chromosomes 2, 3, 5, 8 and 9 in our study are also similar to those reported for a Leghorn x red jungle fowl cross (Rubin et al., 2007).

Whereas the phenotypic correlations between BMD and body weight and AFE are low they are consistent with the expectation that larger and later maturing birds have denser bones. To minimise the effect of oestrogen in influencing QTL for BMD, the NYF, as the main source of oestrogen, was fitted as a covariate. The location and additive effect of the genome-wide significant QTL, after adjusting for the number of yellow follicles, were similar to those of the unadjusted analysis implying that the difference in BMD between the lines due to the influence of this QTL is independent

of oestrogen. Furthermore, none of the QTL identified in this study are located in QTL for yellow follicle numbers (unpublished results).

The confidence intervals for QTL at 131cM on chromosome 1, 297cM on chromosome 2 and 57cM on chromosome 3 overlap with QTL for body weight at several ages in this cross (Sewalem et al., 2002; Podisi et al., 2011). This is not unexpected because large framed individuals are expected to have more tissue mass and therefore stronger bones to support their weight. Furthermore genes controlling weight and size have pleiotropic effects on skeletal traits (Rubin et al., 2007). Nevertheless these and other QTL may harbour genes controlling bone density independently of body weight.

Conclusions

Significant QTL for bone density detected on chromosomes 1, 3 and 8 are similar to QTL for similar traits reported in other studies. The QTL on chromosome 1 and on chromosome 8 do not co-locate with body weight QTL and are likely to harbour genes controlling BMD independently of body weight. Although oestrogen is undoubtedly important to bone density, including the number of yellow follicles did not have a large effect on the power to detect QTL for BMD. The results of this study are consistent with evidence of a genetic basis for the occurrence of osteoporosis and the locations are similar to those of previous results in different breed crosses. Taken together, the results emphasise the importance of these loci in the search for genes and markers for genetic selection to decrease the propensity for osteoporosis and bone fractures in commercial layer hens.

Acknowledgments

The authors gratefully acknowledge the technical assistance of Fiona Pemberton and Graeme Robertson, and the use of UK National Grid Service in carrying out this work. Aviagen Ltd, Newbridge, Midlothian kindly provided the broiler chicks used in these experiments. The research was based on experiments funded by Defra and BBSRC. The Roslin Institute is funded by a core strategic grant from the BBSRC and B.K. Podisi was sponsored by The Government of Botswana.

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Tab.1

The number of microsatellite markers, first and last marker and map length on each linkage chromosome

Chromosome	Number of markers	First marker	Last marker	Map length (cM)
1	17	ROS0008	MCW0107	548
2	13	LEI0163	MCW0157	473
3	15	MCW0169	MCW0037	286
4	4	ADL0317	MCW0180	195
5	5	ROS0013	ADL0298	119
6	4	ADL0323	ADL0142	113
7	3	LEI0064	ADL0180	109
8	9	ROS0021	ROS0075	92
9	4	ROS0078	MCW0134	132
10	1	ADL0209	ADL0209	–
11	5	LEI0110	ROS00112	71
12	2	ADL0240	ADL0044	34
13	2	MCW0340	ADL0225	68
14	1	MCW0123	MCW0123	–
15	2	LEI0083	MCW0080	49
16	1	LEI0258	LEI0258	–
17	1	ADL0199	ADL0199	–
18	2	ROS0022	ROS0027	24
19	1	MCW0094	MCW0094	–
22	1	ROS0073	ROS0073	–
23	1	MCW0249	MCW0249	–
26	2	ADL0285	LEI0074	–
27	1	ROS0071	ROS0071	–
28	3	ROS0095	ADL0299	39
z	6	ROS0072	LEI0075	127
Total	106			2479

Tab.2

Bone mineral density and number of normal yellow follicles from an F_2 broiler-layer cross population at the onset of lay and at 72 weeks of age (mean ± standard deviation and range)

Trait	Mean ± SD	Min	Max
Bird assessed at first egg (N = 388)			
Bone mineral density, mm Al[1)]	1·89 ± 0·38	1·02	3·84
Bone mineral density, ln(mm Al)	0·60 ± 0·19	0·18	1·35
Normal yellow follicles, *N*	12·9 ± 3·3	2	23
Birds assessed at 72 weeks of age (n = 268)			
Bone mineral density, mm Al	2·17 ± 0·46	1·31	4·31
Bone mineral density, ln(mm Al)	0·76 ± 0·20	0·27	1·46
Normal yellow follicles, *N*	5·8 ± 2·1	1	13
Combined population (n = 656)			
Corrected bone mineral density, ln(mm Al)	0·67 ± 0·21	0·18	1·46
Normal yellow follicles, *N*	10·0 ± 4·49	1	23

[1] Millimetre of aluminium density equivalent.

Tab.3

Phenotypic correlations between bone mineral density and reproductive traits at the onset of lay (after laying the first egg) and at the end of lay (72 weeks) in an F_2 broiler-layer cross. All correlations were significantly different from zero at $P<0.05$ unless indicated otherwise (ns)

Trait	BMD at onset of lay	BMD at end of lay
Yellow follicles, n	0·24	0·22
Weight at first egg, g	0·28	0·03^{ns}
Age at first egg, d	−0·18	−0·20
Weight at 72 weeks, g	-	0·42

Tab.4

Quantitative Trait Loci for tibial bone mineral density. Chromosome, chromosome location, F ratio, significance, confidence interval, flanking markers, additive and dominance effects and proportion of variation explained at first egg and 72 weeks of age in an F_2 broiler-layer cross. The analysis of data from the young flock was repeated with a covariate for the number of normal yellow follicles

Chromosome	Position (cM)	F-ratio[1]	CI[2]	Flanking markers	Additive effect ± SE	Dominance effect ± SE	VP[3] (%)
Tibia assessed at first egg, ln(mm Al)							
No covariate, ln(mm Al)							
1	131	5·7†	29–539	LEI0068-LEI0146	0·04 ± 0·01	0·02 ± 0·02	2·3
1	311	9·2*	103–498	LEI0071-LEI0101	0·06 ± 0·02	0·02 ± 0·03	4·1
3	57	7·3†	0–230	HUJ0006-ROS0001	0·04 ± 0·02	−0·08 ± 0·03	3·2
3	187	5·7†	21–213	MCW0252- ADL00306	0·02 ± 0·01	0·06 ± 0·02	2·4
4	65	6·0†	21–243	MCW0295 – ADL0241	0·04 ± 0·01	0·06 ± 0·03	2·5
8	2	9·8*	0–64	ROS0021 – ROS0026	0·04 ± 0·01	−0·03 ± 0·02	4·5
With covariate, ln(mm Al)							
1	131	5·3†	46–533	LEI0068 – LEI0146	0·03 ± 0·01	0·02 ± 0·02	2·1
1	305	10·1**	108–498	LEI0071 – LEI0101	0·07 ± 0·02	0·06 ± 0·04	4·5
3	57	12·6**	52–170	HUJ0006 – ROS0001	0·06 ± 0·02	−0·10 ± 0·03	5·7
3	105	5·3†	24–244	ROS001 – LEI0115	−0·04 ± 0·01	0·0 ± 0·02	2·1
3	194	6·7†	25–246	ADL0306 – ADL0237	0·02 ± 0·01	0·05 ± 0·02	2·8
5	9	7·5†	0–130	ADL0292 – ROS0084	−0·04 ± 0·01	0·06 ± 0·02	3·2
8	2	8·6*	0–81	ROS0021 – ROS0026	0·04 ± 0·01	−0·02 ± 0·02	3·7
Tibia assessed at 72 weeks of age, ln(mm Al)							
No covariate, ln(mm Al)							
2	297	6·3†	12–316	ADL0114-MCW0056	0·06 ± 0·02	0·07 ± 0·04	4·9
8	27	5·3†	0–80	ADL0179-MCW0095	0·05 ± 0·03	0·06 ± 0·03	3·9
With covariate, ln(mm Al)							
2	294	4·9†	12–316	ADL0114-MCW0056	0·05 ± 0·02	0·07 ± 0·04	3·8

[1] Experiment wide significance: *P<0.05, **P <0.01; † chromosome wide suggestive significance.

[2] CI =95% confidence interval.

[3] VP%= percentage of phenotypic variation explained by the QTL.

Tab.5

Quantitative Trait Loci for tibial bone mineral density (loge mm AL equivalent) for the combined data from young (first egg) and old (72 weeks) flocks in an F2 broiler-layer cross. Chromosome, chromosome location, F ratio, significance, confidence interval, flanking markers, additive and dominance effects and proportion of variation explained at first egg and 72 weeks of age

Chromosome	Position (cM)	F-ratio[1]	CI[2]	Flanking markers	Additive effect ± SE	Dominance effect ± SE	VP[3] (%)
1	137	7·2†	119–495	LEI0146 – ADL0319	0·05 ± 0·01	−0·02 ± 0·02	1·9
1	266	5·1†	55–547	LEI0071 – LEI0101	0·01 ± 0·02	0·08 ± 0·02	1·3
8	15	5·9†	0–82	MCW0305 – ADL0258	0·03 ± 0·01	−0·03 ± 0·02	1·5
9	10	7·0†	0–124	ROS0078 – MCW0135	0·04 ± 0·02	−0·06 ± 0·03	1·8

1 † chromosome-wide suggestive significance.

2CI = 95% confidence interval.

3VP% = percentage of phenotypic variation explained by the QTL.