ABSTRACT Genetic selection procedures applied to improve broiler performance may negatively impact the subsequent reproductive efficiency of breeders, particularly in males. Identification of traits that reliably indicate individual male fertility would facilitate selection for reproduction. We hypothesized that physical traits, such as comb area, relative testicular weight, and testicular weight asymmetry, may correlate with fertility in two male-selected primary broiler breeder strains (A and B). Thirty males per strain, individually housed with an average of 10 females, were evaluated at five age periods within the 30-to-50-wk breeding cycle. Flock fertility by candling eggs at Day 19 of incubation and sample fertility by visual assessment of the germinal disc were determined. Sperm penetration (SP) through the perivitelline layer was assessed. Comb area was evaluated by image analysis at 40 and 50 wk, and relative testicular weight was measured at 50 wk. Strain A sample and flock fertility ($P < 0.001$) and SP values ($P < 0.0001$) were significantly lower than Strain B. Both strains had a significant decline of fertility and SP with age ($P < 0.0001$). Strain A comb area correlated with sample fertility ($P < 0.05$), flock fertility ($P < 0.05$), and relative testicular weight ($P < 0.01$). Conversely, Strain B relative testicular weight correlated with sample fertility ($P < 0.0001$) and flock fertility ($P < 0.001$). Significant correlations were not found between testicular weight asymmetry and other reproductive traits. Results suggest comb area may be a reliable indicator of male fertility in Strain A.

(Key words: broiler breeder, fertility, comb area, sperm penetration, testes)

INTRODUCTION

Fertility decline in primary broiler breeder strains is likely attributable to individual males in the flock with extreme subfertility. This differential fertility among males may reduce productivity for the broiler breeder industry (Pollock, 1999). Male broiler breeders must be physiologically and behaviorally mature to successfully elicit the female sexual response and copulate. Fertility problems, particularly those associated with Cornish-derived lines, may result from a physical inability to successfully copulate or from the absence of sexual behavior (Wilson et al., 1979).

Genetic selection focuses on increasing growth rate, body weight, and yield; yet traits related to reproduction may be inadvertently altered in the process. Phenotypic modifications, such as altered secondary sexual characters or reduced libido, may accompany selection for traits of economic importance. These modifications may negatively impact male mating ability, possibly resulting in lower fertility. The impact of genetic selection on reproductive traits and fertility in a natural mating situation remains to be determined.

Semen quality affects the fertility potential of domestic fowl (Cooper and Rowell, 1958; Soller et al., 1965a; Wishart and Palmer, 1986; Lake, 1989; Kirby et al., 1998). For example, artificial insemination (AI) with semen from males subjected to high temperatures resulted in lower fertility and sperm penetration (SP) through the perivitelline layer (PL) overlying the germinal disc (GD) (McDaniel et al., 1995). Fertility and semen quality are often estimated by monitoring the level of SP through the PL, as SP values correlate with fertility and the number of spermatozoa in the female sperm storage tubules (SST) (Brillard and Bakst, 1990; Bramwell et al., 1995).

Males homozygous for the rose comb allele tended to exhibit poor semen quality and subfertility (Froman et al., 1992; Kirby et al., 1994; McLean and Froman, 1996). Although semen quality is relevant to attain high fertility, genetic selection likely has impacts on additional reproductive traits. This effect is particularly important when evaluating fertility in naturally mated broiler breeders.

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Abbreviation Key: AI = artificial insemination; GD = germinal disc; PL = perivitelline layer; SP = sperm penetration; SST = sperm storage tubule.
Most research in broiler breeder fertility uses AI, which removes confounding components associated with natural mating, and is evident, considering the large variation in SP values for eggs collected from naturally mated broiler breeder flocks (Wishart et al., 1992, Wishart and Staines, 1995). Furthermore, fertility under natural mating conditions does not correlate with AI fertility (Van Wambeke et al., 1981) or semen characteristics (Parker, 1961; McCartney and Brown, 1976). Burke and Mauldin (1983) and Jones and Mench (1991) suggested that differential male fertility in natural mating systems is likely due to behavioral differences rather than semen quality, as reproductively successful males must satisfy additional physiological and behavioral requirements.

Behavioral studies in sexual selection of red jungle fowl show female preference for males with larger combs (Zuk et al., 1992, 1995; Ligon and Zwartjes, 1995). Larger combs may reliably indicate roosters with greater semen production (Burrows and Titus, 1939), higher androgen levels (Gilbert, 1971), or increased mating activity (Ligon et al., 1990). Additionally, male comb area may indicate health status. The immunocompetence theory asserts that well-developed secondary sexual characters indicate a male’s ability to avoid parasitization and maintain good health (Hamilton and Zuk, 1982). For example, highly parasitized male red jungle fowl possess smaller combs than healthy males (Zuk et al., 1998). Female preference for males with well-developed secondary sexual characters increases their likelihood of selecting a healthy male in good reproductive condition (Zahavi, 1975; Andersson, 1982, 1984; Kodric-Brown and Brown, 1984; Nur and Hasson, 1984; Pompianowski, 1987; Zuk et al., 1990a, b, 1992, 1995; Möller, 1994a). Hence, male comb area was investigated as a potential fertility correlate in primary broiler breeders.

In addition to comb area, the degree of fluctuating asymmetry may indicate male quality (Møller, 1990). Fluctuating asymmetry refers to the random deviations from symmetry in the development of bilaterally symmetrical traits (Johnstone, 1994; Möller, 1994b), reflecting an individual’s ability to cope with social and environmental stress (Parsons, 1990). Hypothetically, males unaffected by stressors may exhibit less asymmetry, as compared to stressed males. The theory of fluctuating asymmetry suggests more symmetrical males benefit from greater reproductive success than those males exhibiting a high degree of asymmetry (Møller and Swaddle, 1997).

Few researchers have examined broiler breeder fertility in a natural mating system (Burke and Mauldin, 1985; Wishart and Staines, 1995), and the application of sexual selection theory to broiler breeders has not been previously attempted. Sexual selection theory predicts that males with larger combs may elicit a stronger behavioral response in females and, therefore, should successfully initiate and likely complete more matings. Males with higher copulation frequency should achieve higher fertility and SP.

This study evaluated fertility levels, SP values, and morphometric traits of males from two primary broiler breeder strains (A and B) that have undergone different genetic selection regimens. Strain A is Cornish-derived with selection primarily focusing on increasing yield, and all males have rose-type combs. In contrast, Strain B genetic selection focuses on growth rate, and these are single-combed roosters. Annual records historically indicated differential fertility among males, particularly for Strain A. Although Strain A males may potentially be homozygous for the rose comb allele, thus negatively impacting semen quality (Froman et al., 1992), physical traits may correlate with fertility as well. Because previous work by Zuk et al. (1990a, b, 1995) demonstrated that female fowl prefer males with well-developed secondary sexual characteristics, it was hypothesized that male phenotypic traits, such as comb area, testes size, and testicular symmetry, would correlate with male SP and fertility. Once identified, these traits could be incorporated into the genetic selection regimen with the intent of improving fertility.

**METHODS**

**Experimental Design**

This experiment was an initial investigation into the potential relationship between phenotypic traits and fertility of individual males. Two male line genetic strains (A and B) of primary broiler breeders were selected for the study. These birds were part of the routine pureline regeneration procedures implemented by the primary breeder. Males and females were broilerized, which is part of the process for genetic selection, as it requires primary broiler breeders to be initially raised on full feed to allow phenotypic expression of growth rate and yield, thus revealing their market potential. Broilerization provides phenotypic information by which pedigree birds are selected at 7 wk of age. Following selection, these pedigree birds were reared in blackout housing and maintained on a feed-restricted program, although food and water were supplied daily. At 21 wk of age, each male was randomly placed in individual pedigree breeder pens containing an average of 10 females. Males were feed-restricted individually on a daily basis to maintain live weight (3,168 kcal/kg body weight/day and 12% CP). Females were feed-restricted to maintain egg production. Program details are proprietary. Strains A and B were maintained in two different open-side houses on a 16.5 h light:7.5 h darkness lighting program and managed according to standard operating procedures of Perdue Farms, Inc.

Thirty males per strain were selected in a randomized complete block design to account for potential environmental variation within the breeder house. Because males were housed in individual pens, individual males were considered to be the experimental unit of replication, resulting in a sample size of n = 30 per strain. This design was considered sufficient to account for the high inter-individual variability reported in natural mating studies (Wishart et al., 1992). Data were collected on Strains A
and B across the following ages: Period 1 (30 to 33 wk), Period 2 (34 to 37 wk), Period 3 (38 to 41 wk), Period 4 (43 to 46 wk), and Period 5 (48 to 51 wk). At each period, fertility and SP were determined for each experimental male. Comb area was measured at early and late age periods, and testicular weights and live body weights were measured at Period 5. The experimental protocol was approved by the University of Maryland Animal Care and Use Committee.

**Fertility Estimates and PL Sperm Penetration**

Flock fertility was determined by candling all eggs collected daily from the experimental pens, and sample fertility was determined by macroscopic assessment of the GD (Bakst et al., 1997; Hazary et al., 2001) of four fresh-laid eggs collected from each experimental pen at each age period. The SP through the PL was also measured using the sample eggs.

To determine flock fertility, all eggs from the experimental pens were collected four times per day, and sellable eggs were stored for up to 1 wk at 13 C, 75% relative humidity; incubated at 37.5 C, 65% relative humidity, and candled at Day 19 of incubation. Clear eggs, categorized as nonfertile, were not subjected to egg breakout to confirm the nonfertilized state of the GD. Alternatively, sample fertility was estimated by macroscopic examination of the GD of four fresh-laid sample eggs collected per pen per age period. This method provided a more accurate fertility estimate as it allowed differentiation between early dead embryos and nonfertilized GD that are confounded in flock fertility estimates. Sample eggs were stored at 13 C and 75% relative humidity and were evaluated within 5 d of collection. Percentage sample fertility and flock fertility were calculated for individual males at each age period.

Naturally mated broiler breeders respond poorly to manual semen collection, therefore SP rather than direct semen evaluation could more accurately assess semen quality and sperm transfer. After determining fertility of the sample eggs, a 15-mm section of the PL overlying the GD was removed, washed 3X in PBS, and mounted on a Superfrost Plus slide.3 The PL section was fixed with 4% formalin and stained with Schiff’s reagent4 to visualize points of spermatozoal hydrolisis (sperm holes). Holes observed within a 5 × 5-mm eyepiece grid were counted at 40X and reported as the mean number of sperm holes per male (based on the four-egg sample) per age period (Wishart, 1987; Bakst and Cecil, 1997).

**Comb and Testes Measurements**

Male comb area was determined by image analysis5 of digital pictures of the left and right sides of the head taken at Periods 1 and 5 for Strain A and Periods 2 and 5 for Strain B. Scion Image Analysis Software calculates the area of irregular shapes, such as the comb. Each picture, which contained a centimeter ruler for calibration, was opened on a personal computer containing the Scion Image Analysis program. By using the mouse, the perimeter of the comb was traced by the experimenter, and Scion determined comb area. The average of the left and right sides of the comb was calculated, and because comb area did not significantly change with age (P > 0.05), average comb area per male for the early and late periods was analyzed.

At 50 wk (Strain A) and 48 wk (Strain B) of age, males were euthanized according to Perdue Farms’ flock termination schedule. The left (L) and right (R) testes were individually weighed after removal of the epididymal region, and the degree of relative weight asymmetry between the two testes was calculated according to the following formula: |L – R|/(L + R) (Møller and Swaddle, 1997).

**Statistical Analysis**

All data were analyzed with SAS6 software, and P < 0.05 was considered significant. Mean flock fertility, sample fertility, and SP values across age were directly compared between the two strains by mixed-model repeated-measures ANOVA, with age as the repeated measure. Univariate procedures were applied to determine normality and homogeneity of the residual variance in the data sets. Factors in the model were strain, age, and the interaction of the two. Comb area and testes weight relative to body weight met the ANOVA assumptions, and main effects of strain were analyzed by mixed-model ANOVA. For the correlation analyses, sample fertility, flock fertility, and SP values used were based on mean fertility throughout the breeding period per individual male. Pearson parametric correlation analysis was applied individually to Strains A and B to determine the relationships between sample fertility, flock fertility, SP, comb area, relative testes weight, and testicular weight asymmetry.

**RESULTS**

Strain A sample fertility (P < 0.001) and flock fertility (P < 0.001) were lower than the Strain B fertility estimates (Figure 1). Within-strain flock and sample fertility estimates were highly correlated in both Strain A (r = 0.9459; P < 0.0001) and Strain B (r = 0.8648; P < 0.0001). Strain B sample fertility revealed a higher estimate than flock fertility (P < 0.01; Figure 1), a difference not observed for Strain A estimates. Figure 2 illustrates that SP values and percentage sample fertility were highly correlated in Strain A (r = 0.7886; P < 0.0001) and Strain B (r = 0.5690; P < 0.0001). Note that the majority of the nonfertilized eggs contained no sperm holes (82.8% in Strain A and 80.7% in Strain B).

Age negatively impacted sample fertility in both strains (P < 0.0001; Figure 3a). There was a significant main effect of strain (P < 0.0001) on SP values, therefore SP values

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4Sigma Chemical Co., St. Louis, MO 63178.
5Scion Corporation, Frederick, MD 21701.
6SAS Institute, Inc., Cary, NC 27513.
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FIGURE 1. Mean percentage fertility (± SE) for flock and sample fertility estimates.

follow a similar pattern of decline with age as observed with sample fertility \( (P < 0.0001; \text{Figure } 3b) \). Furthermore, a strain \( \times\) age interaction on SP was observed \( (P < 0.001) \), which could be attributed to Period 1, in which SP values did not differ between the strains. However, values diverged at Period 2, in which Strain A had lower SP throughout the rest of the breeding period (Figure 3).

Strain effects on physical traits were found. Strain A had higher relative testes weight \( (P < 0.05; \text{Figure } 4a) \), yet the degree of testicular weight asymmetry did not differ between strains \( (P > 0.05; \text{Figure } 4b) \). Comb area was greater \( (P < 0.0001) \) for Strain B \( (52.8 \pm 1.71 \text{ cm}^2; \text{mean } \pm \text{SE}) \) than for Strain A \( (7.58 \pm 0.24 \text{ cm}^2; \text{mean } \pm \text{SE}) \). Strain A comb area positively correlated with sample fertility \( (r = 0.4140, P < 0.05; \text{Figure } 5a) \), flock fertility \( (r = 0.3962, P < 0.05; \text{Figure } 5b) \), and relative testes weight \( (r = 0.4735, P < 0.01; \text{Figure } 5c) \); however, the relationship between the SP values and comb area was not significant \( (P > 0.05; \text{Figure } 5d) \).

No relationship existed between Strain B comb area and sample fertility, flock fertility, relative testes weight, or SP values \( (P > 0.05; \text{Figure } 5) \). A strong relationship was found between Strain B relative testicular weight and sample fertility \( (r = 0.6453; P < 0.0001) \) and flock fertility \( (r = 0.6056; P < 0.001) \); however, these correlations were not significant \( (P > 0.05) \) for Strain A (Figure 6). Relative testes weight did not significantly correlate with SP values for either strain \( (P > 0.05) \), and testicular weight asymmetry did not significantly correlate with sample fertility, flock fertility, SP, or comb area in either strain \( (P > 0.05) \).

DISCUSSION

The intent of this experiment was to investigate differential fertility in two primary broiler breeder strains and to find phenotypic traits that consistently related to fertility. The focus was on male fertility, because with single-sire pedigree pens, male reproductive failure results in a reduced contribution to regeneration from that male. Subfertile primary broiler breeders may negatively impact reproduction in hybrid broiler breeder strains, which are developed through the generational multiplication process from the primary broiler breeders (Pollock, 1999).

Two fertility estimates were employed in this experiment. Fertility problems were unlikely due exclusively to female impact upon postcopulatory sperm storage and motility, due to the fact that four eggs per pen were sampled at each age period to account for potential female effects. Sample fertility was compared to flock fertility by candling, the standard method for fertility determination by the industry. Compared to Strain B, sample and flock fertility were significantly lower in Strain A. This result follows the historic pattern of greater fertility problems in

FIGURE 2. Correlation between mean sperm penetration values and percentage sample fertility for Strains A and B.
FIGURE 3. a) Sample fertility (mean ± SE) and b) sperm penetration values (mean ± SE) across the five observation periods for Strains A and B. Different letters denote differences between the two strains at a given age period ($P < 0.05$).

FIGURE 4. a) Relative testes weight (mean ± SE) and b) degree of testicular asymmetry (mean ± SE) for Strains A and B. The degree of relative weight asymmetry between the left (L) and right (R) testes was calculated according to the following formula: $|L − R|/(L + R)$ (Møller and Swaddle, 1997).
PL samples containing sperm holes at some point during the breeding period (from 30 to 50 wk of age). Furthermore, visual examination of the ductus deferens and testes upon necropsy indicated that all males appeared reproductively mature and normal. Hence, it appears that infertility was unlikely attributable to inadequate spermatogenesis but, rather, was more likely related to inefficient sperm transfer, possibly through poor mating efficiency (Carte and Leigh-ton, 1969) due to a behavioral or physical incapacity to complete copulations.

Because direct evaluation of semen quality was unattainable for this experiment, semen quality is currently under investigation in a controlled experiment to better establish the relationship between mating behavior, se-

**FIGURE 5.** Correlations between comb area and a) sample fertility, b) flock fertility, c) mean relative testes weight, and d) sperm penetration values.
men quality, and fertility in naturally mating broiler breeders. Furthermore, male mating frequency, which was evaluated for this experiment, is currently being analyzed.

Relationships between phenotype and fertility may provide reliable indicator traits to facilitate the identification and removal of subfertile males from the breeder flock. Comb area in Strain A, which had greater fertility problems, positively correlated with sample fertility, flock fertility, and relative testicular weight. Although a correlation coefficient of 0.4 is low, this number can be interpreted as 16% of the variability in Strain A fertility being explained by comb area. This result appears to account for a reasonable amount of variation, considering that the study was conducted under natural mating conditions. In general, heritability for traits related to fertility tends to be low (Chambers, 1990; Gowe et al., 1993; Leeson and Summers, 2000), yet other reproductive traits, such as testis size, are heritable (Amann, 1999). Selection pressure for comb area in Strain A has the potential to improve fertility in this particular strain of primary broiler breeders, although it should be investigated further before routine application to genetic selection regimes.

These positive correlations suggest that Strain A males with larger combs are more fertile than small-combed males, which is in agreement with Zuk et al. (1990a,b), who found that male red jungle fowl with larger combs were preferred by females and had greater reproductive success. There may be a good cutoff for comb area (e.g., 7 to 8 cm² for Strain A) for which high variation in fertility may still exist, but the chance of selecting subfertile males may be reduced.

Strain A males were potentially homozygous for the rose comb allele and, thus, may have had poor semen quality and subfertility due to this genotype (Froman et al., 1992; Kirby et al., 1994; McLean and Froman, 1996). Although we do not dispute that good semen quality is necessary for obtaining high fertility (Kirby et al., 1998), libido (Ottinger and Mench, 1989), mating frequency, and the physical ability to achieve successful cloacal contact (Wilson et al., 1979; Duncan et al., 1990) are required as well. Our results seem to support the importance of behavioral and physical components, as it appears that Strain A infertility was likely due to poor sperm transfer.

Alternatively, relationships between comb area and reproductive traits for Strain B were undetected, possibly because Strain B comb area was more variable. Furthermore, Strain B males have a single comb, whereas Strain A has a rose comb. Although the rose comb area seems to be a reliable indicator for Strain A fertility, the single comb may provide different visual information, such as comb peak number or comb height. The value of these measurements as fertility predictors remains to be determined.

Strain A comb area was related to fertility, SP, and testes weight. In contrast, Strain B relative testis weight positively correlated with sample and flock fertility; however, this relationship was not found in Strain A. Avian species respond to long-day photoperiods through increased androgen levels and gonadal development (Fol-
lett and Maung, 1978; Balthazart et al., 1981). However, greater testicular weight in broiler breeders may not necessarily correspond to increased spermatogenesis (Janssen et al., 2000) or higher androgen levels (Fernandez-Abella et al., 1999). According to Soller et al. (1965a,b), Cornish males tend to have lower semen quality yet are not significantly subfertile in an AI situation. Hence, semen quality will not likely account for the infertility in the naturally mating flocks we examined. Furthermore, relative testes weight was lower in Strain B, as compared to Strain A, although Strain B had higher fertility. It is possible, however, that once the testes reach a threshold size, relative testicular weight will no longer directly correlate with fertility levels.

For the reasons above, relative testicular weight alone may not predict subfertile males, hence additional behavioral and physical parameters should be evaluated in relation to fertility. For example, it has been demonstrated that house wrens with larger testes at the onset of the breeding season initiate reproductive behavior sooner than males with smaller testes (Evans and Goldsmith, 2000). It is possible that this relationship between relative testicular weight and the rate of copulatory display will be related.

Although testicular weight was not evaluated until the end of the breeding period, this parameter may reliably indicate Strain B fertility. Testes develop to their maximum size upon maturation and are unlikely to regress during the breeding period unless the birds are extremely stressed (Edens, 1983), therefore broiler breeder testicular size potentially relates to physiological reproductive status (Vizcarra et al., 2000). Use of testicular weight as a fertility indicator could be done with the intention of improving the stock of future flocks, as testicular size evaluation is invasive and expensive if conducted at the onset of maturation rather than by necropsy at the end of the breeding cycle.

On the contrary, Strain A relative testes weight was not related to the fertility estimates. Strain A infertility may be due to other factors impacting sperm transfer rather than to inadequate spermatogenesis. Although comb area appears to serve as a reliable fertility indicator for Strain A, a relationship between comb area and semen production has not been established for primary broiler breeders (Burrows and Titus, 1939). If comb development reliably indicates reproductive quality, it may also correlate with behavioral and endocrine factors (Wilson et al., 1979), which are currently being analyzed.

Although fluctuating asymmetry was associated with reproductive success in wild birds (Møller, 1990), testicular weight asymmetry in this study did not relate to any reproductive traits examined. It is possible that the degree of asymmetry in other bilaterally symmetrical physical characteristics (such as testicular size based on length and width measurements, wattle dimensions, or skeletal measurements) may better indicate male quality.

Intensive genetic selection results in divergent strains of broiler breeders that exhibit different behavioral and physiological responses in terms of growth rate, fitness, and reproductive success (Siegel, 1959, 1965, 1972; Chambers, 1990; Barbato, 1999). It is evident that genetic selection played a strong role in altering the physical and physiological components of Strains A and B, as we found contrasting relationships between fertility and phenotypic traits, perhaps related to the specific genetic selection regimes applied to Strains A and B. Although we anticipate that the use of phenotypic fertility indicators in genetic selection is broadly applicable across the broiler breeder industry, these reliable indicators are likely to be specific for any given broiler breeder strain that is examined, depending on the type and intensity of genetic selection the strain is subjected to.

In summary, our results supported the hypothesis that phenotypic correlates of fertility exist. Strain A had lower fertility and SP values; therefore, identification of fertility relationships were particularly relevant for this strain of primary broiler breeders. Because multiple reproductive traits correlated with Strain A comb area, it is suggested that comb area could potentially be a reliable indicator of male reproductive quality for Strain A. If comb area is incorporated into the genetic selection scheme to avoid the incorporation of subfertile males into the breeder flock, fertility may be improved. Strain B indicator traits remain to be revealed.

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