Testicular Growth and Comb and Wattles Development in Three Italian Chicken Genotypes Reared Under Free-Range Conditions

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Testicular growth and comb and wattles development in three Italian chicken genotypes reared under free-range conditions

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Abstract

Male chickens belonging to three Italian purebreds – *Ermellinata di Rovigo* (ER), *Robusta lionata* (RL) and *Robusta maculata* (RM) – were studied. All the birds were reared under the same rearing conditions (from May until autumn). Chickens were reared under infra-red lamps from birth until 4 weeks of age with a 24L:0D photoperiod. Then they were kept outdoor: the photoperiod changed according to the season (from 16L:8D to 12L:12D). At 138 and 168 days of age 20 birds/breed were weighed and then slaughtered. Testicular samples were collected, after evisceration, processed and embedded in paraffine wax. Sections were stained for morphological observations, observed with light microscope, and then classified according to the testis maturation stage. *Ermellinata di Rovigo* chickens showed the lowest (P<0.01) body weight and the highest (P<0.01) testes weight; testes maturity was higher (P<0.01) in ER than in RL, whereas RM was intermediate. For each genotype testes weight and testes maturity did not significantly differ with aging. Correlations between testes weight and body, comb, and wattles weight, according to the breed, were calculated at 168 days of age. For ER no significant correlation was found, whereas RL showed a significant (P<0.01) positive relationship between testes weight and body weight, and sexual secondary characters. *Robusta maculata* showed a significant correlation between testes weight and comb (P<0.01) and wattles weight (P<0.10). Our results suggest that under the studied environmental conditions ER showed the highest testes development according to its more precocious achievement of adult body weight, whereas RL was the least precocious purebred.

Introduction

Northern Italy has a historic tradition for poultry rearing, given the wide areas with cereal crops of the Po river plain. In the past and until 1950, before the incoming and spreading of poultry hybrid genotypes, many poultry purebreds were widely reared in Veneto region. At present only few purebreds are reared and they are very important to preserve biodiversity. Broadly, they are reared under free-range conditions from 4-5 weeks of age, under natural variations of environmental temperature and relative humidity, photoperiod and light intensity. Among environmental parameters, day length and wavelength of light are very important factors for growth and sexual development in birds (Lewis and Morris, 2006; Tyler and Gous, 2012). The avian eyes allow poultry to be responsive to radiation below 400 nm and to see in ultraviolet light; the growth is primarily a response to wavelength, not to intensity, because growth generally decreases with increasing illuminance (Lewis and Morris, 2006). Moreover the efficiency of light in activating the hypothalamus depends not only on the different wavelength but also on the different route made by the light, indirectly via the retina and then optic nerve, or directly through the skull and cranial tissues (Lewis and Morris, 2006).

Another important issue in birds is photorefractoriness, as the inability to respond sexually to an otherwise stimulatory day length: it terminates a breeding season after a period of time on long day lengths, and it is dissipated after exposure to short day lengths (Tyler and Gous, 2012). In fact, birds which are not restricted on feeding and exposed to about two months of short photoperiods (as in nature, by short winter day length) dissipate this condition; in this process T_4 hormone is involved. Alternatively, photorefractoriness can be stopped by pharmacologically induced hypothyroidism or by markedly reducing illuminance when birds are held on long days (Lewis and Morris, 2006). A consequence of bird photorefractoriness is seasonality: offspring are hatched when the most favourable environmental conditions occur to maximize the chances of survival. Partridge needs an exposition to short days for photorefractoriness to be dissipated (Siopes and Wilson, 1978), but some species dissipate photorefractoriness even if they are kept on long days, with a dissipation occurring at a slower rate (Tyler and Gous, 2012). In broiler breeders with no previous exposure to short day lengths sexual maturity is delayed (Lewis *et al.*, 2004). Age of sexual maturity is influenced by body weight, but the flock response to photostimulation can be variable, despite the uniformity in the target body weight (Lewis and Morris 2006; Lewis *et al.*, 2007). In commercial hybrid genotypes, when a flock is photostimulated, a bimodal frequency distribution of age at maturity results from the advance in maturity of some birds and the delay in others (Tyler and Gous, 2012). Tyler and Gous (2012) stated that the response to early photostimulation is heritable. Lewis *et al.* (2007) suggested that there are some genetic variations in the acquisition of photosensitivity. Photorefractoriness has been demonstrated in the male of the avian species, in starlings and in bantams of *Gallus domesticus*, but not in broiler breeders (Sreekumar and Sharp, 1998; Tyler and Gous, 2012).

Gender differences in response to photoperiod exist as the reproductive control mechanisms have evolved differently between males and females, according to the geographical position of the species and the sexual and parental behaviour (Ball and Ketterson, 2008).

A few information exists on Italian chicken genotypes concerning the growth rate and the carcass characteristics (Rizzi *et al.*, 2007, 2009a, 2009b). These local genotypes have a slower body growth compared to the commercial hybrids (Rizzi *et al.*, 2009a, 2009b, 2013). For these purebreds no information is available on the physiological responses, such as gonadal growth and sexual maturity age.

Materials and methods

Animals and rearing conditions

Male chickens belonging to three Italian purebreds – *Ermellinata di Rovigo* (ER), *Robusta lionata* (RL) and *Robusta maculata* (RM) – were reared. These breeds are dual-purpose genotypes of Northern Italy, of Veneto region; they were created in 1950 using Sussex and Rhode Island for ER, Brown Orpington and White America for RL and RM. The trial started in May and lasted at the end of October. From birth all the males (40 chickens per each breed) were reared in heterosexual groups under the same rearing conditions (environmental temperature, feeding, photoperiod and light intensity). The chickens were reared indoor on wood shavings and under infra-red lamps, on continuous illumination from 1 day until 4 weeks of age (average temperature decreasing from 33 to 24°C), then the photoperiod was natural. From the 5th week of age the birds were kept outdoor, under free-range conditions, on pasture; the environmental conditions widely changed throughout this period: the temperature (Figure 1) and the relative humidity (Figure 2) ranged from about 30 to 10°C and from 60 to 85%, respectively. The photoperiod (Figure 3) changed according to the season in boreal hemisphere (from 16L:8D to 12L:12D).

A single diet was used for the free-range period (crude protein= 22.0%; ether extract=4.9%; crude fibre=3.6%; ash=6.8%) and the feed was administered *ad libitum*. At 138 and 168 days of age the birds (20 males/breed) were weighed and then slaughtered after electrical stunning.

Testes measurements and histological analyses

At 138 and 168 days of age testicular samples were collected, immediately after the removal of the intestines. The testes were weighed to the nearest 0.01 g, fixed in 10% formalin, dehydrated through a graded series of ethanol and embedded in paraffine wax. Serial sections (10 μm) were stained with Haematoxylin-Eosin and Azan Mallory for morphological observations. The slides were examined under light microscope and the testes were classified according to the testis maturation stage [testis maturity index (TMI)] as follows: Class 0: *prepubere* testis, a simple single layer of cells (spermatogonia and Sertoli cells) in the seminiferous tubules, abundant interstitial space; Class 1: 2 layers of cells in the seminiferous tubules, primary spermatocytes (cells with darkly stained chro-
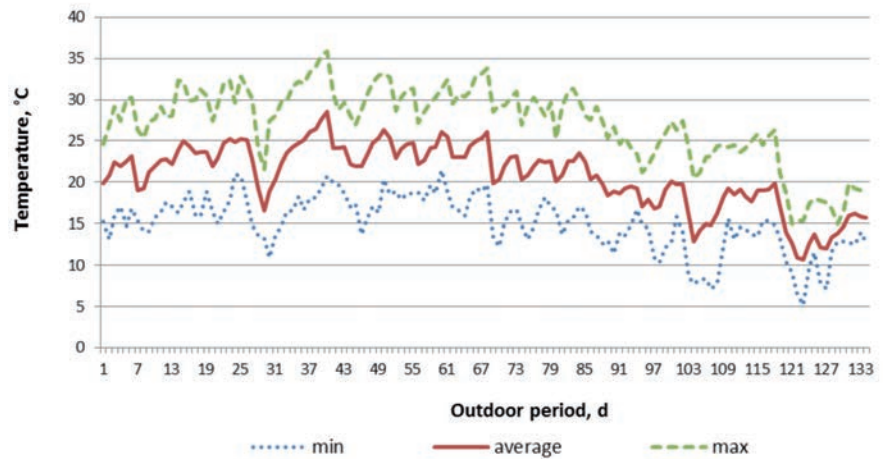

Figure 1. Environmental temperature throughout the outdoor period (the chickens were kept outdoor from 32 days of age).
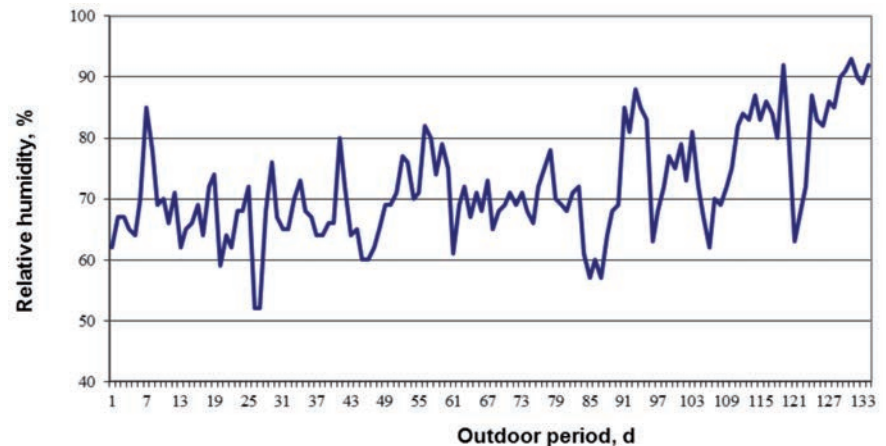

Figure 2. Environmental relative humidity throughout the outdoor period (the chickens were kept outdoor from 32 days of age).
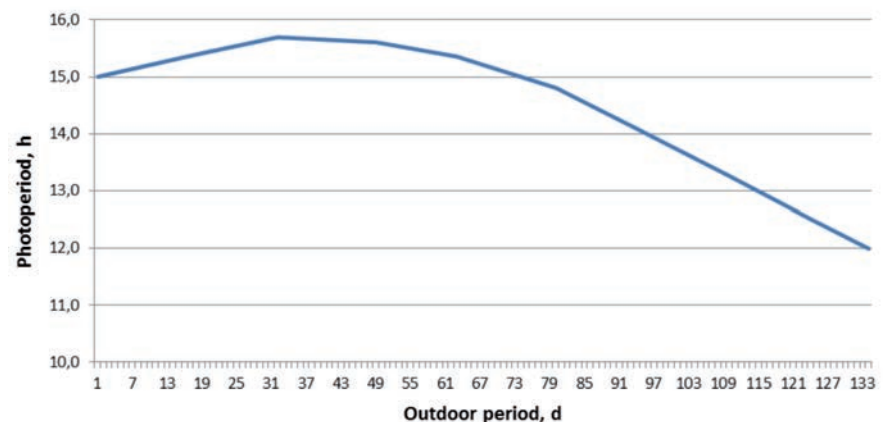

Figure 3. Photoperiod (hours of light) throughout the outdoor period (the chickens were kept outdoor from 32 days of age).

matin) moving away from the basement membrane; Class 2: 3-4 layers of cells in the seminiferous tubules; Class 3: 5-6 layers of cells in the seminiferous tubules, presence of sperm cells demonstrating the spermatogenic activity; Class 4: more than 7 layers of cells in the seminiferous tubules, presence of numerous sperm cells demonstrating the active spermatogenesis in the lumen, reduction of interstitial tissue.

Comb and wattles measurements

After slaughtering at 168 days of age, comb (single comb) and wattles were excised from the head of the birds and weighed to the nearest 0.01 g; comb and wattles from 7 birds of each genotype were measured (length and height) by callipers to the nearest 0.1 mm.

Statistical analysis

Data on body weight (BW), % of adult BW, testes weight and TMI were subjected to 2-way ANOVA with breed and age as main effects; on the same data a 1-way ANOVA was used to test the effect of age by breed (SAS, 2010). Comb and wattles data were subjected to 1-way ANOVA with breed as main effect (SAS, 2010). Duncan's multiple range test was performed when significant differences were found (SAS, 2010). On data of testes weight, BW, comb weight and wattles weight Pearson correlations were calculated (SAS, 2010).

Results and discussion

Live body weight and gonadal characteristics

As far as the breed effect is concerned, from 138 until 168 days of age ER chickens showed the lowest (P<0.01) BW compared to the RL and RM males (Table 1). An opposite trend was observed for the % of adult BW which was higher (P<0.01) in ER than in RL and RM. *Ermellinata di Rovigo* showed higher (P<0.01) testes weight than RL and RM; the TMI was higher (P<0.01) in ER than in RL, whereas in RM it was intermediate.

With regard to the effect of age according to the breed (Table 2), only RL showed significant increases (P<0.05) of live BW and % of adult BW, whereas for the other two breeds the body gain rose at a not significant and weaker extent. For each genotype, the testis weight and the TMI did not significantly differ with age.

On the basis of literature indications on the adult BW of these breeds (Veneto Agricoltura,

2012) the ER males reached more precociously the adult BW compared to the other breeds as they reached 79% of adult BW at 138 days of age, whereas RL and RM reached 78 and 74% of adult BW, respectively, 30 days later. As regards infra-red illumination carried out on the first weeks of age as warming source, we did not observe any macroscopic eye abnormalities or other pathologies or an abnormal mortality rate as indicated by Lewis and Morris (2006). These authors stated that in continuously illuminated birds the severity of the eye abnormalities was markedly reduced after the birds were transferred to natural lighting (Lewis and Morris, 2006).

The testes weight in broiler breeders constitutes about 1% of the total BW, with values ranging from 9 to 30 g per testis at sexual maturity, depending on the breed (Sturkie and

Opel, 1976). The breeds tested exhibited a lower testes weight and % of the total BW (0.5% in ER and 0.1% in RL and RM) than broiler strains; this observation is in agreement with data reported by Oke and Ihemenson (2010) in a study on Nigerian local cocks and by Baeza *et al.* (2009) in Geline de Touraine genotype chickens reared outdoor. Indications on relationships between testes weight and semen volume and concentration, total number of sperms and sperm mobility are controversial (Froman and Feltmann, 1998; Lee *et al.*, 1999; Zaniboni and Cerolini, 2008). However, there is a wide variability in testes both for weight and for spermatozoa production within a breed (Zaniboni and Cerolini, 2008).

Observations on the testis parenchyma highlighted that at the age of 138 days sper-

Table 1. Effect of breed on body weight and gonadal development of male chickens from 138 to 168 days of age.

	Breed			SE
	ER	RL	RM	
BW, g	2697[Bb]	3211[Aa]	3127[Aa]	55
Adult BW, %	83[Aa]	76[Bb]	74[Bb]	1
Testes weight, g	12.6[Aa]	4.76[Bb]	4.88[Bb]	1.3074
TMI	3.42[Aa]	2.30[Bb]	2.88[AaBb]	0.2678

ER, *Ermellinata di Rovigo* (12 males); RL, *Robusta lionata* (20 males); RM, *Robusta maculata* (17 males); BW, body weight; TMI, testis maturity index. [A,B]Mean values within a row followed by different letters are significantly different (P<0.01); [a,b]mean values within a row followed by different letters are significantly different (P<0.05).

Table 2. Effect of age on body weight and gonadal development from 138 to 168 days of age.

	Breed		
	ER	RL	RM
BW, g			
138 d	2564	3009[b]	3020
168 d	2791	3319[a]	3163
Adult BW, %			
138 d	79	71[b]	72
168 d	86	78[a]	74
Testis weight, g			
138 d	4.54	2.56	0.91
168 d	7.57	2.29	2.95
TMI			
138 d	3.20	2.57	2.00
168 d	3.57	2.16	3.17
	SE		
BW	73	83	85
% Adult BW	2.2527	1.7792	1.9943
Testis weight	1.6974	0.5616	0.7704
TMI	0.4231	0.3222	0.4361

ER, E*rmellinata di Rovigo* (5-7 males); RL, *Robusta lionata* (7-13 males); RM, *Robusta maculata* (5-12 males); BW, body weight; TMI, testis maturity index. [a,b]Mean values within a column followed by different letters are significantly different (P<0.05).

matogenesis started in most of the birds of the three breeds. In the seminiferous tubules, at least 2 layers of cells lied between basal lamina and lumen. These features denoted the beginning of spermatogenic activity. Nevertheless variability was observed among birds regarding the spermatogenesis phase; this variability was due both to the intrinsic characteristics of the single individual and to the pecking order, the hierarchical system of social organization in chickens (Guhl, 1956; Mench and Keeling, 2001). Literature stated that the dominant males exhibited the development of primary and secondary sexual characters, while the other males showed a sexual development inhibition (Guhl, 1964; Zuk *et al.*, 1990; Parker and Ligon, 2003; Cornwallis and Birkhead, 2008). At the age of 168 days the histological analyses of the testis parenchyma pointed out the spermatogenic activity. Sexual development appeared at a more advanced level than at the first age. Generally, the birds appeared not to be affected by the reduction of photoperiod with the exception of RL, which showed a generally negative trend regarding testicular development. Indeed, at the second age testis weight and TMI of RL were slightly lower than at the first age, and the testis was less active. Among the three breeds studied, ER resulted the strain that reached sexual maturity earlier. This breed exhibited evident phase of spermiation, occurring at the end of spermatogenesis, when spermatozoa are in the apical part of Sertoli cells from which they are released into the lumen of seminiferous tubules, into the fluids secreted by Sertoli cells (Zaniboni and Cerolini, 2008).

From 138 to 168 days of age, in ER a marked testis weight increase was observed whereas the TMI showed a less marked increase. *Robusta maculata* showed a marked increase in the testis weight and in the TMI, even if no significant effect was observed. Furthermore, ER showed a rapid growth (about 100 mg/d), which occurs in *pubere* males (Zaniboni and Cerolini, 2008). In chicken males, under a constant photoperiod of 16L:8D, pubertal age occurs when mature spermatozoa are visible, at about 10 until 12 weeks of age, whereas adult testes weight is reached at 20 weeks, and within 22 weeks the gonadal growth definitively stops (Zaniboni and Cerolini, 2008). The start of a rapid testis growth phase can change according to the genotype and to the environmental conditions, in particular to the photoperiod (Tyler and Gous, 2009, 2012). Thyroid hormones are involved in gonadal processes, thus interaction between temperature and testis growth could be considered. As far as photoperiod is concerned, when the day lenght

induces an earlier phase of rapid growth of the testis, the differentiation phase of Sertoli cells also occurs earlier, thus Sertoli cells interrupt their proliferation process; this fact causes a lower final volume of the testis (Zaniboni and Cerolini, 2008). Tyler and Gous (2009) ascertained that the fastest testis growth phase may not necessarily be accompanied by the production of functional spermatozoa, and this aspect is possibly delayed until the testis has reached its mature weight. Indications on pullets stated that growing birds respond more to a change in photoperiod than to the initial or final photoperiods themselves; another important factor affecting the timing of sexual maturation is the age at which the day length is changed (Lewis and Morris, 2006).

Light information received by both the retina and the hypothalamus - with differences in retinal sensitivity, in penetrability through the skull and the cranial tissues and in sensitivity of hypothalamic photoreceptors - strongly influences the gonadal growth, which also varies with wavelength. The exposure to white or red lights allowed achieving the greatest stimulation for sexual maturation in male domestic birds (Lewis and Morris, 2006). Red and white light, when compared to blue or green light, have a strong influence on sexually mature domestic cockerels as a consequence of a more stimulatory effect of longer wavelengths of light (Harrison *et al.*, 1970). Comparing the effect of red, blue, green and white light and two day lengths (6 and 14-h photoperiod), only in cockerels reared under red or white 6-h photoperiod, lower testes weight and inhibitions of spermatogenesis and gonadotropin release were observed at 18 weeks of age (Lewis and Morris, 2006).

Furthermore, another environmental factor affecting male sexual maturity is the rearing of males along with females; literature indicates that the social stimulation, as the presence of females, stimulate the gonadal devel-

Table 3. Effect of breed on comb and wattles weight and size at 168 days of age.

	Breed			SE
	ER	RL	RM	
Comb weight, g	7.62[ABb]	6.20[Bb]	9.93[Aa]	0.6317
Wattles weight, g	5.98[ABa]	4.69[Bb]	6.36[Aa]	0.4266
Comb length, cm	7.56[ABa]	6.62[Bb]	8.27[Aa]	0.2879
Comb height, cm	3.66[ABa]	3.00[Bb]	4.10[Aa]	0.1735

ER, *Ermellinata di Rovigo* (observations: 17 weight; 7 length and height); RL, *Robusta lionata* (observations: 17 weight; 6 length and height); RM, *Robusta maculata* (observations: 18 weight; 7 length and height). [AB]Mean values within a row followed by different letters are significantly different (P<0.01); [a,b]mean values within a row followed by different letters are significantly different (P<0.05).

Table 4. Correlations between body, comb and wattles, and testes weight at 168 days of age according to the breed.

	ER		
	Body weight	Comb weight	Wattles weight
Comb weight	ns	-	ns
Wattles weight	ns	0.62 (P<0.008)	-
Testes weight	ns	ns	ns
	RL		
	Body weight	Comb weight	Wattles weight
Comb weight	0.47 (P<0.04)	-	ns
Wattles weight	0.46 (P<0.04)	0.91 (P<0.0001)	-
Testes weight	0.64 (P<0.004)	0.79 (P<0.0001)	0.84 (P<0.0001)
	RM		
	Body weight	Comb weight	Wattles weight
Comb weight	ns	-	ns
Wattles weight	ns	0.87 (P<0.0001)	-
Testes weight	ns	0.58 (P<0.01)	0.45 (P<0.06)

ER, *Ermellinata di Rovigo* (observations: 17 body, comb and wattles weight; 9 testes weight); RL, *Robusta lionata* (observations: 20 body, comb and wattles weight; 18 testes weight); RM, *Robusta maculata* (observations: 18 body, comb and wattles weight; 18 testes weight); ns, not significant.

opment in the dove (O'Connel et al., 1981) and in Japanese quail (Delville et al., 1984). The interaction between social factors and the light has to be considered (Cheng, 1976).

Comb and wattles characteristics

Table 3 summarizes the comb and wattles weight and their size according to the breed. Robusta maculata showed the highest (P<0.01) weight and dimensions of comb and wattles, RL showed the lowest (P<0.01) values and ER was intermediate. Ermellinata di Rovigo comb weight was lower (P<0.05) than RM, whereas ER wattles weight was higher (P<0.05) than RL; ER comb length and height were higher (P<0.05) than RL.

Table 4 shows correlations between testes weight and body, comb, and wattles weight according to the breed, at 168 days of age. The results differed among the groups. For ER no significant correlation was found, whereas RL showed a significant positive relationship between testes weight and BW (P<0.01) and sexual characters (P<0.01). Robusta maculata showed significant correlations only between testes weight and comb (P<0.01) and wattles weight (P<0.10). The comb and wattles weight were significantly (P<0.01) correlated in the three breeds. The BW was correlated (P<0.05) to the comb and the wattles weight only in RL.

Comb and wattles showed different sizes among the breeds. This result does not confirm the testes development status among the groups as comb and wattles size is a breed-specific character. The incidence of comb and wattles on BW showed a trend among the breeds slightly different from that observed for the testes (ER=0.50%; RL=0.32%; RM=0.51% vs ER=0.50%; RL=0.14%; RM=0.20%). Our results indicate that the comb and the wattles weight are correlated with testes weight only in the breeds in which the body growth (skeletal and muscle tissue, data not shown) has not yet finished, but environmental factors and/or their interaction with genotype could be involved.

In domestic fowl, females often use a male's comb or wattles as a signal for male quality and prefer to mate with males with larger and redder appendages (Zuk et al., 1992, 1995; Ligon and Zwartjes, 1995). Comb size and testosterone level are positively correlated (Allee et al., 1939; Collias, 1943; Witschi, 1961), while the relationship between comb size and sperm quality in the domestic chickens is controversial. A positive relationship between comb size and sperm quality seemed to be strain specific (Gebriel et al., 2009; El Ghany et al., 2011; Udeh et al., 2011). Some studies reported no relationship between comb size and sperm quality (Pizzari et al., 2004; Bilcik and Estevez, 2005; Baczynski, 2008; Prieto et al., 2011), while others reported a negative relationship between comb size and sperm movement (Parker et al., 2006; Navara et al., 2012).

Conclusions

This study is a first contribution to the knowledge of some physiological traits of three Italian chicken breeds. The results indicate that from 20 to 24 weeks of age the three breeds studied showed different BW and testicular growth. At 24 weeks of age, under the environmental conditions examined, the testes weight was correlated to the sexual accessory characters in RL and RM. At this age the development of the sexual accessory characters stopped in ER, whereas in RL and RM still continued. Robusta maculata showed an intermediate status as the testes weight was not yet correlated to the BW but it was correlated to the comb weight and to the wattles weight, even if at a weaker extent than in RL.

Actually, these Italian breeds are reared following seasonal cycles, thus a reproductive phase in the same year of birth could be possible only for those birds born in the first months of the year, according to genotype. In fact the results indicate a more precocious body development and, consequently, sexual maturation of ER, whereas RM and RL are progressively less precocious. This result partially agrees with the only indication available on these breeds (Veneto Agricoltura, 2012) that reports a similar maturity age (from 6 to 7 months) for the three breeds considered. For a selection phase in a breeding programme, genotype, birth date and photoperiod have to be considered. In our experimental conditions, the selection of birds at 168 days of age towards earlier sexual development by means of comb or/and wattles weight evaluation seems to be possible for RL and RM but not for ER.

However, for a good management of these dual-purpose breeds more investigation is needed for achieving economic purposes and for preserving the animal welfare. The lighting programme (wavelength, photoperiod and illuminance) throughout the first weeks of life is not standardized as in the commercial poultry farming, so the chicks are exposed to a wide range of lighting conditions that could differently affect their gonadal development. In a breeding programme of purebred chickens reared under free-range conditions, to select males according to the reproductive traits (for a good semen production, elimination of ster-

ile or sub-fertile birds) genotype and photoperiod have to be seriously considered, since selection could occur at different ages (from 20 to 24 weeks of age), as stated by Zaniboni and Cerolini (2008), according to breed and environmental conditions. Therefore, more knowledge is needed to elucidate the most favourable solution given that an interaction between these factors and genotype could occur and affect the chicken physiological responses.

Finally, for dual-purpose purebreds, environmental light and sexual maturity could be considered also for meat quality as these two factors could affect sensorial and chemical parameters of chicken meat (Touraille and Ricard, 1981; Kim et al., 2013).

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