Nondestructive characterization gender of chicken eggs by odor using SPME/GC-MS coupled with chemometrics

Xiao-le Xiang,[*,†] Gan Hu,[*] Yong-guo Jin,[*] Guo-feng Jin,[*] and Mei-hu Ma[*,1]

[*]*National Research and Development Center for Egg Processing, College of Food Science and Technology, Huazhong Agricultural University, Wuhan, Hubei 430070, PR China; and* [†]*School of Food Science and Bioengineering, Changsha University of Science and Technology, Changsha, Hunan, PR China*

ABSTRACT It's a difficult task for researchers to identify the gender of chicken eggs by nondestructive approach in the early of incubation, which not only could reduce the cost of incubation, but also could improve the welfare of chicks. Therefore, SPME/GC-MS has been applied to investigate its potential as a nondestructive tool for characterizing the differences of odor between male and female chicken eggs during early of incubation and even before hatch. The results showed that more volatiles were found in female White leghorn eggs during early of incubation and 6,10-dimethyl-5,9-undecadien-2-one, 6-methyl-5-hepten-2-one, nonanal, decanal, octanal, 2-nonen-1-ol, etc. were important for the distinction of male and female White leghorn eggs during E_1-E_9 of incubation. 2-ethyl-1-hexanol; octanal, nonanal, 2,2,4-trimethyl-3-carboxyisopropyl pentanoic acid isobutyl ester; 2-nonen-1-ol, cyclopropanecarboxamide, heptadecane were correlated with gender of unhatched White leghorn, Hy-line brown and Jing fen eggs, respectively. Moreover, sex-related volatiles have been strongly influenced by incubation process and egg breed, and to be related to steroid hormone biosynthesis. What's more, this study enables us to develop a new visual for ovo sexing of chicken eggs and advances our understanding of the biological significance behind volatiles emitted from chicken eggs.

Key words: chicken eggs, sex-related volatiles, ovo sexing, nondestructive characterization

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INTRODUCTION

The increasing specialization of chicken lines for meat and egg production has made male and female chicks are used for broiler and layer strains, respectively (Galli et al., 2017). More than 7.0 billion freshly hatched cockerels with unwanted gender, therefore, were culled globally annually, especially for male day-old chicks in commercial hatcheries (Alin et al., 2019). Which not only cause significant economic losses but also raise serious ethical issues (Galli et al., 2017). Under these pressure, there is urgent need for new techniques of sex determination “in ovo” during early of incubation (Galli et al., 2017, 2018).

Nowadays, many minimally invasive and/or nondestructive techniques have been used to detect the gender of embryo in ovo during early of incubation or even in unhatched fertilized eggs (Alin et al., 2019). For example, the concentration of hormonal (estrogen) in allantoic fluid (Weissmann et al., 2013) and reflectance spectroscopy both provided good sexing results at the mid period of incubation (Rozenboim and Ben Dor, 2001). Infrared and optical spectroscopy have been applied for sexing of unhatched eggs by addressing the DNA content extracted from blastoderm cells (Steiner et al., 2011; Galli et al., 2018; Wu et al., 2019). Raman and fluorescence spectral information of blood from embryo through eggshell membrane at day 3.5 of incubation ($\mathbf{E_{3.5}}$) for ovo sexing with a correct rate up to 90 and 93%, respectively (Galli et al., 2018). In addition, the shape and color of eggs have been proposed related to their sex (Aviles et al., 2011; Yilmazdikmen and Dikmen, 2013).

However, all of the above methods require hatched eggs to be opened with a shell windowing, which will strongly affects hatching rate and chick health in future, and not easy to be exploited in practice (Galli et al., 2018). Therefore, many researchers have attempted to apply nondestructive strategies to solve this problem and spectroscopy have been considered as the most promising technologies until now. For example, hyperspectral spectroscopy has been successfully used to identify the gender of unhatched eggs(Ngadi et al., 2018). But the spectral features acquired from unhatched eggs could not provide

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[1]Corresponding author: mameihuhn@163.com

enough valid information to characterize the differences between male and female fertilized eggs.

Fortunately, an increasing number of researches have focused on the roles of odor or olfaction for sex recognition in avian (Caro et al., 2015; Costanzo et al., 2016). More importantly, it was surprisingly found that there were certain differences in odor profiles between male and female Japanese quail eggs both at E_8 and E_1 (Webster et al., 2015). What's more, sex-related variation in odor of eggs were also found in wild barn swallow at $E_{10\text{-}11}$ (Costanzo et al., 2016). Hence, it could suspected that there may be certain difference in volatiles between unhatched male and female fertilized eggs. In ovo sexing of chicken eggs by odor not only has the potential to enables nondestructive testing but also provides more detailed information for mechanism (Caro et al., 2015; Costanzo et al., 2016).

It is widely accepted that sex-specific differences in metabolites between male and female embryos were existed in the middle and later of incubation, due to sex differentiation (Smith and Sinclair, 2004; Weissmann et al., 2013). However, it is difficult to understand sex differences in violates and spectral characteristics of hatched eggs were existed at E_1 or even before hatched, except for genetic information (Webster et al., 2015; Ngadi et al., 2018). In fact, as far as we know, the starting point of sex determination and/or differentiation for avian embryo could be advanced to meiosis I (Uller and Badyaev, 2009), cell-autonomous mechanisms of somatic sex identity and sex-based differences in steroid hormones derived from maternal investment could support it indirectly (Radder, 2007).

From the above, sex-specific volatiles and spectral features have begun to be realized in quail, barn swallow eggs and fertilized chicken eggs, respectively. However, to our knowledge, no researches have reported on the sex-specific volatiles of chicken eggs till now. The present study, thus, was designed to characterize the composition and differences of odor emitted from male and female chicken eggs during early of incubation and then to further evaluate the variation between unhatched male and female chicken eggs. Gender detection of unhatched fertilized chicken eggs by odor would improve productivity of hatcheries, beneficial for animal welfare and offers the potential for industrial exploitation in future.

MATERIALS AND METHODS

Fertilized Eggs Storage and Incubation

Freshly fertilized chicken eggs, including white Leghorn (**W**), Hy-line brown (**H**), and Jing fen (**J**), were obtained from a commercial supplier (Wuhan, Hubei province, China) and stored in room temperature until hatch at 38°C and 60% humidity in an incubator (Fuhui Tech Co., Wuhan, China).

SPME-GC-MS

Acquisition of volatiles from hatched eggs were performed using 50/30 μm DVB/CAR/PDMS (Supelco, Bellefonte, PA) following the protocol in Xiang (Xiang et al., 2019).

- GC: The VOCs enriched from chicken eggs were desorbed in GC injector at 250°C for 5 min in a splitess mode with a helium (99.99%) flow rate of 1.0 mL/min and separated on a HP-5MS capillary column (30 m × 0.25 mm × 0.25 mm film thickness) using 7890B-5977A GC-MS instrument (Agilent Technologies, Santa Clara, CA). GC oven was programmed from 30°C for 2 min, increased to 45°C at 2°C min^{-1}, and increased to 120°C at 3°C min^{-1} (hold 2 min), finally increased to 230°C at 6°C min^{-1} and maintained for 5 min. Quantitative datas of VOCs were semiquantified by peak areas of in the selected ion monitor (**SIM**).
- MS: Temperatures of ion source and quadrupole were 230°C and 150°C, respectively. Quadrupole mass spectrometer was operated in EI mode at 70 eV and scan range was set at m/z 35−450. Tentative VOC identification was performed by NIST 11.0 Mass-Spectral Search Library and RI (Xiang et al., 2019).

Molecular Sexing

DNA from embryos in each egg were extracted using DNA tissue Kit (Sangon Biotech, Shanghai, China) following manufacturers' protocols. PCR amplification was run using primers SF (5'-GTGCATTGCAGAAGCAATATT-3') and SR (5'-GCCTCCTGTTTATTATAGAATTCAT-3'). About 25 μL systems were used: 1.5 μL (10 μmol/L) of both primers, 8.5 μL Red Master Mix (Sangon Biotech), 1.5 μL extracted DNA, and 1.5 μL H_2O. PCR assay conditions were set at 94°C for 5 min followed by 35 cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 40 s and a final extension step of 72°C for 7 min. PCR reactions were performed using a T100 Thermal Cycler PCR (Bio-Rad, Hercules, CA). PCR products were separated on 1.8% agarose gels at (120 V, 15 mA) and visualized with 4S Green Nucleic Acid Stain and UV light. One and two band indicated male and female egg, respectively (Galli et al., 2018).

Statistical and Bioinformatics Analyses

All statistical and bioinformatics analysis were performed by IBM SPSS 24 and Metabo Analyst 4.0, respectively.

RESULTS AND DISCUSSION

Differences in VOCs Between (Hatched) Male and Female Eggs (W) During E_0−E_9

Fouteen fertilized eggs were used for data acquisition during E_0−E_7, 5 eggs were identified as male and female

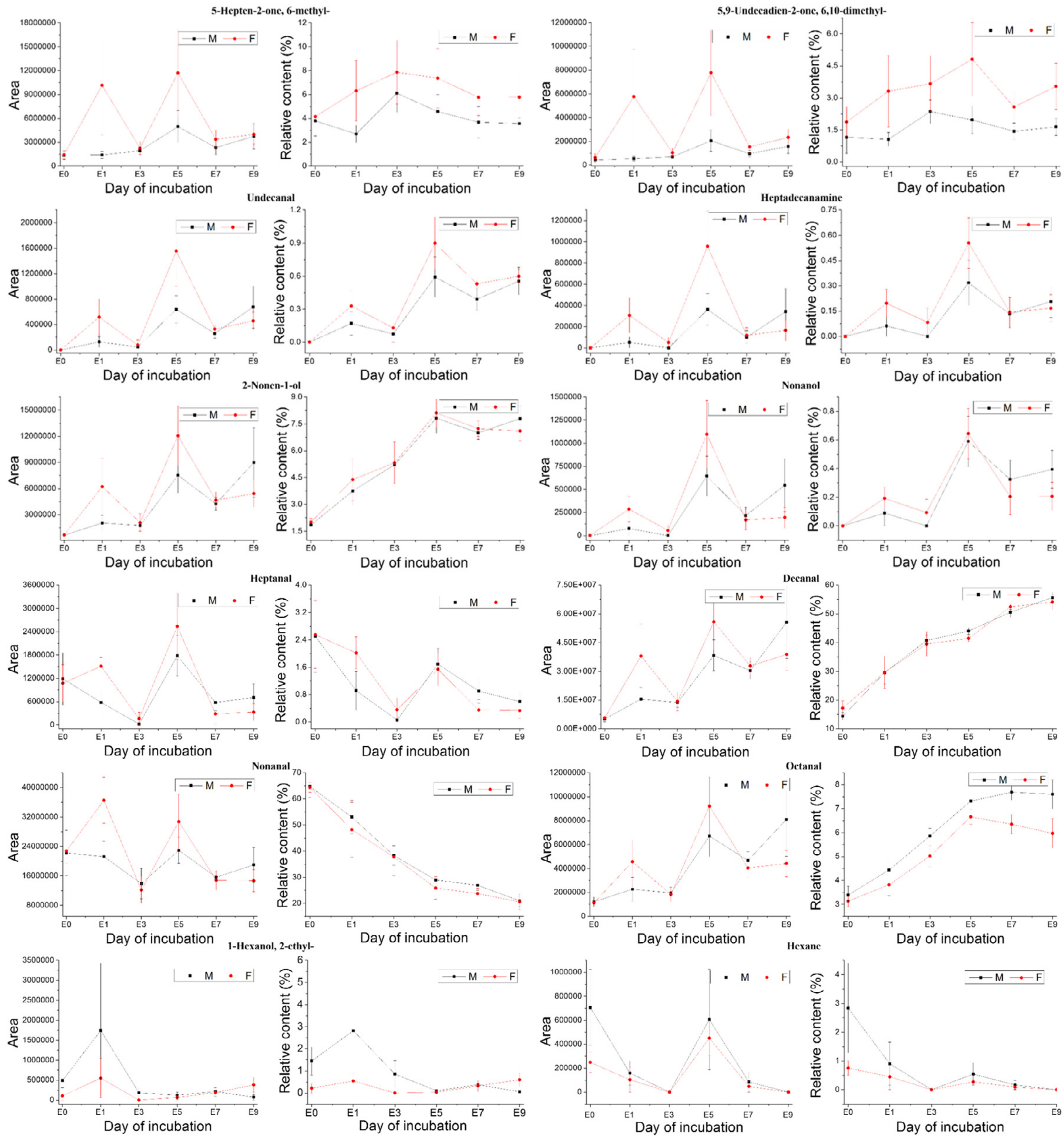

Figure 1. Sex difference in concentration of VOCs emitted from chicken eggs during E_0−E_9 of incubation (Mean ± SE; M: male, F: female; left: area, right: percentage).

eggs, respectively and the rest 4 eggs were infertile or sex were not sure. Thirteen embryo eggs (6 male and 7 female) were used for data acquisition during E_9 (Figure S1). The weight of these fertilized eggs had no significant difference.

Comparison Analysis A total of 18 VOCs were identified in hatched eggs (Figure 1 and Table 1), including: 7 aldehydes (hexanal, heptanal, octanal, nonanal, decanal, undecanal, dodecanal); 3 alcohols (2-ethyl-1-hexanol, 1-nonanol, 2-nonen-1-ol); 2 ketones (6-methyl-5-Hepten-2-one, 6,10-dimethyl-5,9-Undecadien-2-one); 2 alkanes (n-hexane, 2-fluoro-7-hydroxybicyclo[2.2.1] heptane), 1-heptadecanamine, 3-(bromomethyl)-piperidine, cedrene and carbon dioxide. Most of these VOCs have been reported in hatched quail, barn swallow eggs (Webster et al., 2015; Costanzo et al., 2016) and fertilized chicken eggs (Xiang et al., 2019). The abundance of almost VOCs emitted from female eggs were higher than that from male eggs during early of incubation and obvious difference in VOCs were obtained at E_1 and E_5 (Figures 1, S2 and Table 1). Similar results were obtained in barn swallow and quail hatched eggs (Webster et al., 2015; Costanzo et al., 2016) and which might be due to sex difference in embryonic metabolism or selective utilization of egg components (Martins, 2004).

It's clear that the average levels of 6,10-dimethyl-5,9-undecadien-2-one, 6-methyl-5-hepten-2-one, undecanal, heptadecanamine, 2-nonen-1-ol, nonanal, etc. emitted from female eggs were higher than that from male eggs and the opposite result was obtained for 2-ethyl-1-hexanol, hexane, etc (Figure 1). But the difference of VOCs between male and female eggs was not statistically significant, except for the abundance of heptanal, nonanal and cedrene at E_1 and the percentage of octanal at E_7 and E_9 (Table 1). As well known, saturated aldehydes have usually been considered as the derivative of lipid

Table 1. Mean levels of VOCs between male and female eggs for W breed during E_0−E_9 of incubation (mean ± SE).

E₀	RT min	Volatile compounds	M (n = 5)	F (n = 5)	M (n = 5)	F (n = 5)
	2.67	n-Hexane	707,289 ± 314,503	248,720 ± 86,168	2.84 ± 1.56	0.76 ± 0.25
	7.50	Hexanal	1,919,745 ± 1,586,849	1,293,390 ± 959,108	2.66 ± 1.24	2.81 ± 1.90
	12.56	Heptanal	1,191,947 ± 670,059	1,073,318 ± 471,558	2.51 ± 1.06	2.55 ± 0.99
	17.31	6-methyl-5-Hepten-2-one	1,376,781 ± 521,867	1,418,052 ± 511,202	3.79 ± 1.26	4.15 ± 1.19
	18.08	Octanal	1,225,262 ± 395,234	1,127,781 ± 201,276	3.39 ± 0.37	3.14 ± 0.24
	19.59	2-ethyl-1-Hexanol	491,138 ± 177,650	108328 ± 108328	1.45 ± 0.64	0.22 ± 0.22
	23.37	Nonanal	22,245,370 ± 6,172,755	22,751,195 ± 3,261,170	64.77 ± 4.16	64.42 ± 1.90
	26.63	2-Nonen-1-ol	655,662 ± 195328	699,271 ± 107,457	1.85 ± 0.28	2.01 ± 0.19
	28.35	Decanal	5,131,160 ± 1,492,170	5,695,401 ± 463,820	14.37 ± 1.18	17.21 ± 2.47
	39.52	6,10-dimethyl-5,9-Undecadien-2-one	424,690 ± 223,201	640,914 ± 290,067	1.16 ± 0.75	1.88 ± 0.69
	43.83	Cedrene	330,705 ± 131,457	245,510 ± 101,300	1.04 ± 0.48	0.64 ± 0.22
E₁	RT min	Volatile compounds	M (n = 5)	F (n = 5)	M (n = 5)	F (n = 5)
	2.67	n-Hexane	159,031 ± 100,907	103,603 ± 103,603	0.90 ± 0.75	0.45 ± 0.45
	7.50	Hexanal	211,689 ± 145,803	240,690 ± 91,426	0.40 ± 0.31	0.31 ± 0.14
	12.56	**Heptanal**	**579,731 ± 356,533**	**1,511,187 ± 227,597**	0.91 ± 0.57	2.02 ± 0.47
	17.31	6-methyl-5-Hepten-2-one	1,401,011 ± 444,627	10,177,035 ± 6,259,544	2.70 ± 0.73	6.31 ± 2.54
	18.08	Octanal	2,276,164 ± 998,216	4,567,335 ± 1,858,499	4.44 ± 0.71	3.82 ± 0.45
	19.59	2-ethyl-1-Hexanol	1,744,661 ± 1,681,903	550,671 ± 487,749	2.82 ± 2.75	0.55 ± 0.36
	21.55	1-Nonanol	76,388 ± 76,388	286,406 ± 137,757	0.09 ± 0.09	0.19 ± 0.08
	23.37	**Nonanal**	**21,234,819 ± 4,173,891**	**36,532,779 ± 6,215,023**	53.04 ± 6.43	48.21 ± 10.53
	26.63	2-Nonen-1-ol	2,059,868 ± 1,016,308	6,217,979 ± 3,264,844	3.75 ± 0.81	4.38 ± 1.17
	28.35	Decanal	15,499,005 ± 6,221,383	38,023,189 ± 1,673,7899	29.59 ± 3.93	29.7 ± 5.61
	31.69	1-Heptadecanamine	52,249 ± 52,249	306637 ± 165,645	0.06 ± 0.06	0.20 ± 0.08
	33.03	Undecanal	127,234 ± 83,870	517,315 ± 285,712	0.17 ± 0.11	0.33 ± 0.14
	39.52	6,10-dimethyl-5,9-Undecadien-2-one	546,348 ± 194,957	5,769,678 ± 4,001,630	1.06 ± 0.32	3.32 ± 1.67
	43.83	**Cedrene**	**0 ± 0**	**127,584 ± 53,664**	**0.00 ± 0.00**	**0.12 ± 0.07**
E₃	RT min	Volatile compounds	**M (n = 5)**	F (n = 5)	M (n = 5)	F (n = 5)
	7.50	Hexanal	136,108 ± 136,108	8,573 ± 8,573	0.21 ± 0.21	0.02 ± 0.02
	12.56	Heptanal	22,306 ± 13,693	161,951 ± 161,951	0.06 ± 0.04	0.36 ± 0.36
	17.31	6-methyl-5-Hepten-2-one	1,946,654 ± 480774	2,260,972 ± 806,622	6.11 ± 1.59	7.87 ± 2.66
	18.08	Octanal	1,961,132 ± 330,221	1,840,817 ± 598,321	5.86 ± 0.32	5.01 ± 0.44
	19.59	2-ethyl-1-Hexanol	184,280 ± 119,468	0 ± 0	0.86 ± 0.61	0.00 ± 0.00
	21.55	1-Nonanol	0 ± 0	56,443 ± 56,443	0.00 ± 0.00	0.09 ± 0.09
	23.37	Nonanal	13,939,047 ± 4,144,008	12,186,148 ± 3,664,919	38.3 ± 3.75	37.78 ± 7.25
	26.63	2-Nonen-1-ol	1,754,216 ± 315,068	2,101,424 ± 992,929	5.22 ± 0.25	5.33 ± 1.16
	28.35	Decanal	13,695,073 ± 2,566,530	14,279,402 ± 4,950,148	40.73 ± 1.89	39.54 ± 4.24
	31.69	1-Heptadecanamine	0 ± 0	50313 ± 50,313	0.00 ± 0.00	0.08 ± 0.08
	33.03	Undecanal	47,313 ± 47,313	78,244 ± 78,244	0.07 ± 0.07	0.13 ± 0.13
	39.52	6,10-dimethyl-5,9-Undecadien-2-one	711,989 ± 109,937	1,046,693 ± 377,237	2.36 ± 0.54	3.67 ± 1.30
E₅	RT min	Volatile compounds	M (n = 5)	F (n = 5)	M (n = 5)	F (n = 5)
	1.71	Carbon dioxide	189,757 ± 76,733	432,268 ± 105,489	0.2 ± 0.08	0.35 ± 0.05
	2.67	n-Hexane	607,043 ± 419,218	451,448 ± 146,844	0.54 ± 0.39	0.27 ± 0.10
	7.50	Hexanal	1,143,704 ± 756,053	2,140,233 ± 1,235,922	1.05 ± 0.70	1.11 ± 0.65
	12.56	Heptanal	1,783,603 ± 525,191	2,541,099 ± 866,840	1.69 ± 0.46	1.54 ± 0.47
	17.31	6-methyl-5-Hepten-2-one	4,967,512 ± 2,026,371	11,723,078 ± 5,384,954	4.57 ± 1.42	7.37 ± 2.49
	18.08	Octanal	6,711,175 ± 1,712,218	9,216,594 ± 2,420,535	7.32 ± 0.46	6.66 ± 0.31
	18.52	2-Fluoro-7-hydroxybicyclo[2.2.1] heptane	154,326 ± 94,957	65,748 ± 65,748	0.13 ± 0.08	0.04 ± 0.04
	19.59	2-ethyl-1-Hexanol	124,393 ± 76,295	65,800 ± 56,879	0.10 ± 0.06	0.04 ± 0.03
	21.55	1-Nonanol	644,152 ± 214,165	1,096,920 ± 365,662	0.59 ± 0.17	0.64 ± 0.18
	23.37	Nonanal	22,932,633 ± 3,518,122	30,722,671 ± 7,490,784	28.97 ± 3.59	25.86 ± 4.3
	26.63	2-Nonen-1-ol	7,519,158 ± 2,034,952	12,056,957 ± 3,435,050	7.82 ± 0.84	8.11 ± 0.80
	28.35	Decanal	38,283,684 ± 8,082,834	55,841,642 ± 14,470,657	44.03 ± 1.16	41.46 ± 1.1
	31.69	1-Heptadecanamine	361,857 ± 149,036	957,676 ± 336,104	0.32 ± 0.13	0.55 ± 0.15
	33.03	Undecanal	639,752 ± 214,755	1,553,224 ± 550,442	0.59 ± 0.18	0.9 ± 0.24
	35.97	Piperidine, 3-(bromomethyl)-	0 ± 0	194,289 ± 133,152	0.00 ± 0.00	0.09 ± 0.06
	37.76	Dodecanal	49,621 ± 49,621	246,576 ± 112,988	0.05 ± 0.05	0.14 ± 0.06
	39.52	6,10-dimethyl-5,9-Undecadien-2-one	2,052,258 ± 896,894	7782,720 ± 3,599,126	1.97 ± 0.64	4.81 ± 1.71
	43.83	Cedrene	88,889 ± 54,605	114,224 ± 114,224	0.08 ± 0.05	0.06 ± 0.06
E₇	RT min	Volatile compounds	M (n = 5)	F (n = 5)	M (n = 5)	F (n = 5)
	1.71	Carbon dioxide	192,127 ± 72,367	110,692 ± 63,363	0.35 ± 0.15	0.23 ± 0.13
	2.67	n-Hexane	84,345 ± 84,345	47,631 ± 40,581	0.17 ± 0.17	0.09 ± 0.08
	7.50	Hexanal	48,193 ± 48,193	0 ± 0	0.08 ± 0.08	0.00 ± 0.00
	12.56	Heptanal	577,464 ± 216,950	286,001 ± 263,128	0.90 ± 0.36	0.35 ± 0.31
	17.31	6-methyl-5-Hepten-2-one	2,334,341 ± 857,860	3,369,616 ± 1,098,751	3.68 ± 1.31	5.76 ± 1.52
	18.08	**Octanal**	4,665,762 ± 739,104	4,045,272 ± 755,074	***7.69 ± 0.33***	***6.35 ± 0.40***
	19.59	2-ethyl-1-Hexanol	214,313 ± 111,207	185,777 ± 125,744	0.38 ± 0.19	0.32 ± 0.25
	21.55	1-Nonanol	217,082 ± 90,686	170,146 ± 106,019	0.32 ± 0.13	0.20 ± 0.13
	23.37	Nonanal	1,5638,615 ± 1,447,539	14,833,732 ± 2,656,896	26.9 ± 1.91	23.74 ± 2.04
	26.63	2-Nonen-1-ol	4,256,260 ± 730,177	4,668,091 ± 912,702	7.00 ± 0.37	7.23 ± 0.43
	28.35	Decanal	3,0409,966 ± 4,495,900	32,828,397 ± 4,585,357	50.57 ± 1.74	52.48 ± 0.91
	31.69	1-Heptadecanamine	100,277 ± 62,097	118,989 ± 72,902	0.13 ± 0.08	0.14 ± 0.09
	33.03	Undecanal	260,347 ± 71,743	328,412 ± 49,381	0.39 ± 0.1	0.53 ± 0.03
	39.52	6,10-dimethyl-5,9-Undecadien-2-one	966,082 ± 283,944	1,543,351 ± 553,621	1.43 ± 0.39	2.58 ± 0.76
E₉	RT min	Volatile compounds	M (n = 6)	F (n = 7)	M (n=6)	F (n = 7)
	1.71	Carbon dioxide	442,090 ± 123,026	474,897 ± 175,688	0.70 ± 0.31	0.90 ± 0.39
	7.50	Hexanal	259,849 ± 120,719	121,785 ± 53,356	0.21 ± 0.10	0.16 ± 0.08
	12.56	Heptanal	706,175 ± 354,736	329,054 ± 216,066	0.60 ± 0.29	0.34 ± 0.23
	17.31	6-methyl-5-Hepten-2-one	3,748,807 ± 1,596,740	3,992,474 ± 1,206,013	3.57 ± 0.74	5.78 ± 1.74
	18.08	**Octanal**	8,105,079 ± 3,076,097	4,420,044 ± 1,112,527	**7.60 ± 0.61**	**5.97 ± 0.61**
	19.59	2-ethyl-1-Hexanol	74,164 ± 74,164	380,747 ± 183,959	0.06 ± 0.06	0.60 ± 0.30
	21.55	1-Nonanol	545,431 ± 287,063	196,731 ± 110,800	0.40 ± 0.13	0.21 ± 0.10
	23.37	Nonanal	18,990,678 ± 4,810,172	14,688,386 ± 2,985,200	21.00 ± 2.33	20.55 ± 3.03
	26.63	2-Nonen-1-ol	8,988,117 ± 3,970,659	5,424,999 ± 1,539,190	7.79 ± 0.72	7.10 ± 0.55
	28.35	Decanal	55,721,327 ± 1,896,6737	38,823,219 ± 8,226,992	55.68 ± 1.91	54.07 ± 2.59
	31.69	1-Heptadecanamine	342,342 ± 214,986	165,149 ± 98,250	0.21 ± 0.10	0.17 ± 0.08
	33.03	Undecanal	676,199 ± 330,365	456,383 ± 140,090	0.56 ± 0.13	0.60 ± 0.06
	39.52	6,10-dimethyl-5,9-Undecadien-2-one	1,584,791 ± 594,151	2,337,356 ± 684,490	1.64 ± 0.40	3.54 ± 1.09

Bold: $0.05 < P < 0.1$; bold and italic: $P < 0.05$.

oxidation degradation and Strecker reaction of amino acid (Mir et al., 2017; Xiang et al., 2019; Jia et al., 2020).

Multivariate Analysis Trend of sex differences in odor emitted from eggs has been preliminarily discovered during early of incubation and multivariate analysis was then used to visualize the differences between male and female eggs (Xiang et al., 2019). As expected, the VOCs emitted from male and female eggs during early of incubation (E_1-E_9) were separated in 2D score plots of OPLS-DA model, except for E_0 and E_5 (Figure 2). However, a clear difference (or trend) of VOCs between eggs with either sex were shown in 3D score plots (Figure S3). It suggests that there are indeed some subtle differences between VOCs emitted from male and female chicken eggs. Minor differences between VOCs profile for male and female eggs was obtained at E_5 may be caused by the surge in metabolic activity of

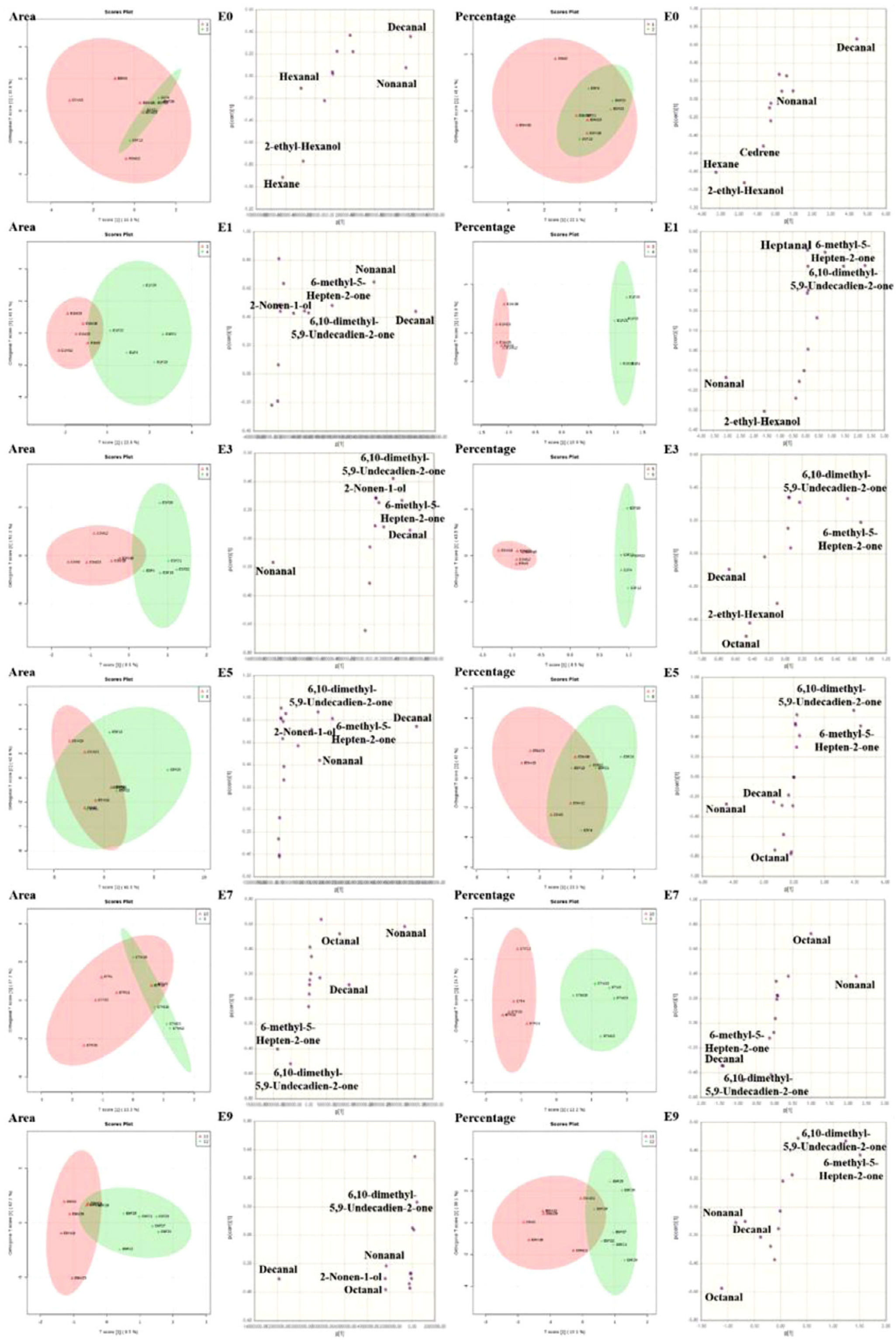

Figure 2. Sex difference of VOCs emitted from male and female chicken eggs by OPLS-DA during E_0-E_9 of incubation.

embryo. Which may expanded the variation of VOCs emitted from eggs and then the difference between VOCs emitted from male and female eggs has been relatively concealed (Bruggeman et al., 2002; Ayers et al., 2013).

Six,10-dimethyl-5,9-undecadien-2-one, 6-methyl-5-hepten-2-one, nonanal, decanal, octanal, 2-nonen-1-ol, etc. were important for the distinction of male and female eggs during E_1-E_9 (Figure 2). Moreover, most of these VOCs were more abundant in female eggs, except for 2-ethyl-1-hexanol and hexane. Similarly, many ketones, acids, alcohols and aldehydes have been reported more abundant in female eggs (Webster et al., 2015; Costanzo et al., 2016). For instance, methylheptenone has been reported to be more abundant in female organisms and been considered as biological relevant odors for rats' erection (Curran et al., 2007; Nielsen et al., 2013). What's more, female ostriches has been reported more sensitive to 6-methyl-5-hepten-2-one than male ostriches (Sole et al., 2010) and 2,2,6-trimethylcyclohexanone was identified as female-specific compounds (Li and Zhang 2018). On the contrary, 2-heptanone and 6,10-dimethyl-5,9-undecadien-2-one have been reported as male-specific pheromone compounds (Ayers et al., 2013; Mayo et al., 2013). It can be inferred that sex-pheromone ketones may be affected by many factors, including species, environment and so on.

Moreover, aldehydes have been identified as the main pheromone volatiles for chicken eggs (Xiang et al., 2019) and sex difference in aldehydes have been found in rabbit meat, *Parasitoid, Bracon hebetor Say* and olive fly (Botsi et al., 1995; Dweck et al., 2010; Xie et al., 2016). For example, nonanal has been considered as a minor sex-pheromone for olive fly (Botsi et al., 1995) and been proved to exert higher influence in females during oviposition period (Malheiro et al., 2015). Unsaturated nonen-1-ol has been reported produced by male *Anastrepha ludens* to atteact conspeific females (Nation, 1983).

Discriminant and Correlation Analysis VOCs emitted from male and female eggs during early of incubation (E_0−E_9) were well separated in canonical discriminant (**CD**), except for one unhatched male egg was misjudged as female egg. In other words, the accuracy of (W) egg sexing during E_1 −E_9 of incubation was almost 100% (Figure 3 and Table S1). It was very interesting and lucky that VOCs emitted from female eggs were always located at the upper or left of male eggs (Figure 3). Furthermore, hexanal, heptanal, octanal, 6-methyl-5-hepten-2-one, 1-nonanol, etc. and octanal, nonanal, 2-ethyl-1-hexanol, 1-nonanol, etc. were greater contribution on the distinction of male and female eggs during E_1 and E_3 (Table S2). Hexanal showed a significant difference between male and female *Leptolossus zobatus* (Inoue et al., 2019) and heptanal could reduce its sensitivity to the peripheral and central olfactory level independently of mating status (Deisig et al., 2012). Hexanal, heptanal, and nonanal were also reported have the potential to attract female *T. infestans* (Fontan et al., 2002).

While carbon dioxide, heptanal, 2-fluoro-7-hydroxybicyclo[2.2.1] heptane, etc. carbon dioxide, hexanal, decanal, undecanal, etc. and octanal, 6,10-dimethyl-5,9-undecadien-2-one, 2-ethyl-1-hexanol, 1-nonanol, etc. were greater contribution on the distinction of male and female eggs during E_5, E_7, and E_9, respectively (Table S2). It is well accepted that the difference of carbon dioxide between eggs from both sexes may be resulted from differential metabolism of male and female embryo in eggs (Martins, 2004). Coincidentally, more alcohols were also detected in male starlings during mating and breeding (Amo et al., 2012), such as nonanol was only found in male *Trupanea vicina* abdomen and released from pleural glands to influence the female's receptivity for mating attempts (Kosi et al., 2013).

In addition, the relationship between VOCs emitted from hatched eggs with their sex was further assessed by correlation analysis. Nonanal, cedrene (area, $0.01 < P < 0.05$), heptanal (area, $0.05 < P < 0.1$) and carbon dioxide (area, $0.01 < P < 0.05$), heptadecanamine, undecanal (area, $0.05 < P < 0.1$) were significantly positively correlated with gender of eggs during E_1 and E_5, respectively. Six,10-dimethyl-5,9-undecadien-2-one (area, $0.05 < P < 0.1$), octanal (percentage, $0.01 < P < 0.05$), and octanal (percentage, $0.05 < P < 0.1$) were significantly negatively correlated with gender of eggs during E_7 and E_9, respectively (Table 2). Cedrene could be selectively bonded and transported by CmedPBP4, which exhibited

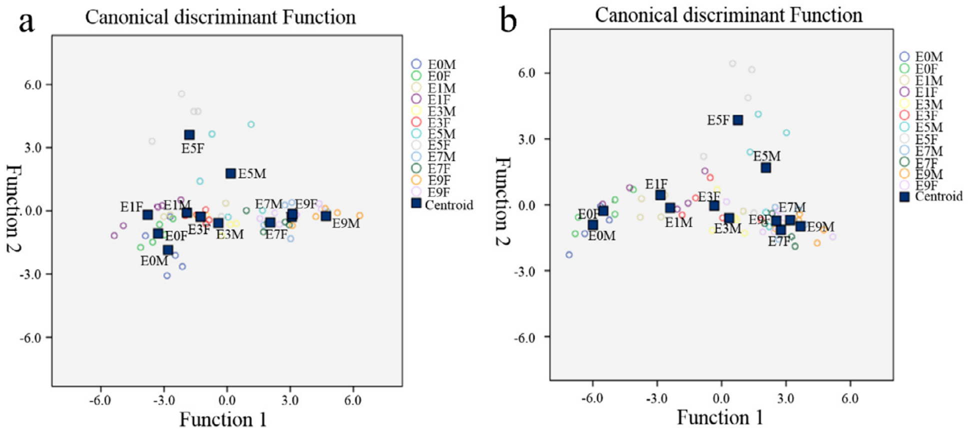

Figure 3. Scatter plot of VOCs emitted from male and female chicken eggs during E0-E9 of incubation by canonical discriminant analysis.

Table 2. Correlation between sex and VOCs emitted from chicken eggs (W) during E_0–E_9.

RT min	Volatile compounds	E_0				E_1				E_3			
		Area		Percentage		Area		Percentage		Area		Percentage	
		R	p	R	p	R	p	R	p	R	p	R	p
2.67	n-Hexane	−0.453	0.189	−0.453	0.189	−0.129	0.723	−0.214	0.552				
7.50	Hexanal	−0.104	0.774	−0.104	0.774	0.244	0.496	0.175	0.629	−0.050	0.892	−.050	.892
12.56	Heptanal	0.070	0.848	−0.070	0.848	**0.594**	**0.070**	0.384	0.273	−0.129	0.723	−0.129	0.723
17.31	6-methyl-5-Hepten-2-one	0.104	0.774	0.105	0.773	0.313	0.378	0.314	0.376	0.035	0.924	0.433	0.244
18.08	Octanal	0.174	0.631	−0.035	0.924	0.313	0.378	−0.244	0.497	−0.174	0.631	−0.383	0.275
19.59	2-ethyl-1-Hexanol	**−0.557**	**0.094**	**−0.631**	**0.050**	0.000	1.000	0.000	1.000	−0.497	0.144	−0.497	0.144
21.55	1-Nonanol					0.431	0.213	0.279	0.435	0.333	0.347	0.333	0.347
23.37	Nonanal	0.313	0.378	−0.174	0.631	***0.661***	***0.037***	0.104	0.774	−0.174	0.631	−0.035	0.924
26.63	2-Nonen-1-ol	0.035	0.924	0.174	0.631	0.313	0.378	0.035	0.924	−0.244	0.497	−0.383	0.275
28.35	Decanal	0.313	0.378	0.244	0.497	0.313	0.378	−0.035	0.924	−0.174	0.631	−0.244	0.497
31.69	1-Heptadecanamine					0.510	0.132	0.431	0.213	0.333	0.347	0.333	0.347
33.03	Undecanal					0.409	0.241	0.334	0.345	0.050	0.892	0.050	0.892
39.52	6,10-dimethyl-5,9-Undecadien-2-one	0.176	0.626	0.176	0.626	0.349	0.323	0.349	0.323	0.244	0.497	0.244	0.497
43.83	Cedrene	−0.349	0.323	−0.140	0.700	***0.643***	***0.045***	***0.643***	***0.045***				
		E_5				**E_7**				**E_9**			
1.71	Carbon dioxide	***0.661***	***0.037***	0.419	0.228	−0.247	0.492	−0.247	0.492	0.000	1.000	0.000	1.000
2.67	n-Hexane	0.106	0.771	0.106	0.771	0.129	0.723	0.129	0.723				
7.50	Hexanal	0.000	1.00	−0.070	0.848	−0.333	0.347	−0.333	0.347	−0.195	0.523	−0.065	0.833
12.56	Heptanal	0.140	0.700	−0.245	0.495	−0.317	0.372	−0.388	0.268	−0.259	0.394	−0.188	0.538
17.31	6-methyl-5-Hepten-2-one	0.313	0.378	0.244	0.497	0.279	0.434	0.419	0.228	0.082	0.789	0.247	0.415
18.08	Octanal	244	0.497	−0.313	0.378	−0.174	0.631	***−0.731***	***0.016***	−0.330	0.271	**−0.536**	**0.059**
18.52	2-Fluoro-7-hydroxybicyclo [2.2.1] heptane	−0.300	0.400	−0.300	0.400								
19.59	2-ethyl-1-Hexanol	−0.157	0.665	−0.157	0.665	−0.037	0.919	−0.111	0.759	0.353	0.237	0.353	0.237
21.55	1-Nonanol	0.140	0.700	0.000	1.000	−0.111	0.759	−0.224	0.535	−0.347	0.245	−0.390	0.187
23.37	Nonanal	0.313	0.378	−0.174	0.631	−0.174	0.631	−0.313	0.378	−0.289	0.339	−0.165	0.590
26.63	2-Nonen-1-ol	0.383	0.275	0.000	1.000	0.035	0.924	0.104	0.774	−0.082	0.789	−0.103	0.737
28.35	Decanal	0.383	275	−0.453	0.189	0.104	0.774	0.313	0.378	−0.124	0.687	−0.082	0.789
31.69	1-Heptadecanamine	**0.599**	**0.067**	0.424	0.222	0.157	0.665	0.118	0.745	−0.179	0.558	−0.112	0.715
33.03	Undecanal	**0.559**	**0.093**	0.419	0.228	0.104	0.349	0.419	0.228	−0.041	0.894	0.000	1.000
35.97	3-(bromomethyl)-Piperidine	0.497	0.144	0.497	0.144								
37.76	Dodecanal	0.431	0.213	0.394	0.260								
39.52	6,10-dimethyl-5,9-Undecadien-2-one	0.383	0.275	0.349	0.323	**−0.301**	**0.055**	0.419	0.228	0.247	0.415	0.372	0.211
43.83	Cedrene	−0.129	0.723	−0.129	0.723								

R: Spearman's correlation coefficient, p: significance value.
Bold: $0.05 < P < 0.1$; bold and italic: $P < 0.05$.

different expression levels and showed obvious antenna-specific expression patterns between sexes (Sun et al., 2016).

More importantly, it is noticed that the variation and correlation between VOCs and sex of chicken eggs were strongly influenced by incubation time. Hexane, 2-ethyl-1-hexanol, decanal, cedrene, etc. were greater contribution on the distinction of unhatched male and female eggs and 2-ethyl-1-hexanol (area, $0.05 < P < 0.1$; percentage, $P = 0.05$) were significantly negatively correlated with gender of unhatched fertilized eggs. Furthermore, the potential role of other sex-related pheromone alcohols, including 2-ethyl-1-hexanol, 1-octanol, etc., are not yet clear (Levi-Zada et al., 2013; Webster et al., 2015).

Differences in VOCs Between Unhatched Male and Female Eggs for W, H, J Breed

Based on the above findings, 69 H and 60 J unhatched fertilized eggs were used for datas acquisition to explore the difference between VOCs from *unhatched* male and female eggs together. Thirty five and 29 fertilized eggs (H) were identified as male and female eggs, the rest 5 H eggs were infertile or sex were not sure; 26 fertilized eggs (J) were both identified as male and female eggs, the rest 8 J eggs were infertile or sex were not sure (Figure S1). The weight of fertilized eggs for H and J breed had no significant difference.

Comparison Analysis A total of 27 VOCs were identified in unhatched fertilized eggs, among them, 11, 14, and 20 VOCs in W, H, and J eggs, respectively (Figure 4 and Table 3). There were certain variation in absolute abundance and relative content of each VOC between male and female eggs and no significant difference in common VOCs (both for area and percentage) were found between male and female eggs for 3 breeds (W, H, and J) (Figure 4 and Table 3). But, some sex-specific VOCs were found in eggs for each breed, for instance, the concentration (area and percentage) of nonanal was significant different between unhatched male and female H eggs ($0.05 < P < 0.1$); the percentage of pentanoic acid, 2,2,4-trimethyl-3-carboxyisopropyl, isobutyl ester and 6,10dimethyl-5,9-undecadien-2-one emitted from female H eggs were found to be higher than that from male H eggs ($0.05 < P < 0.1$). While the percentage of octanal, 2-nonen-1-ol, decanal ($0.05 < P < 0.1$) and cyclopropanecarboxamide, heptadecane ($P < 0.05$) were different between male and female J eggs and the

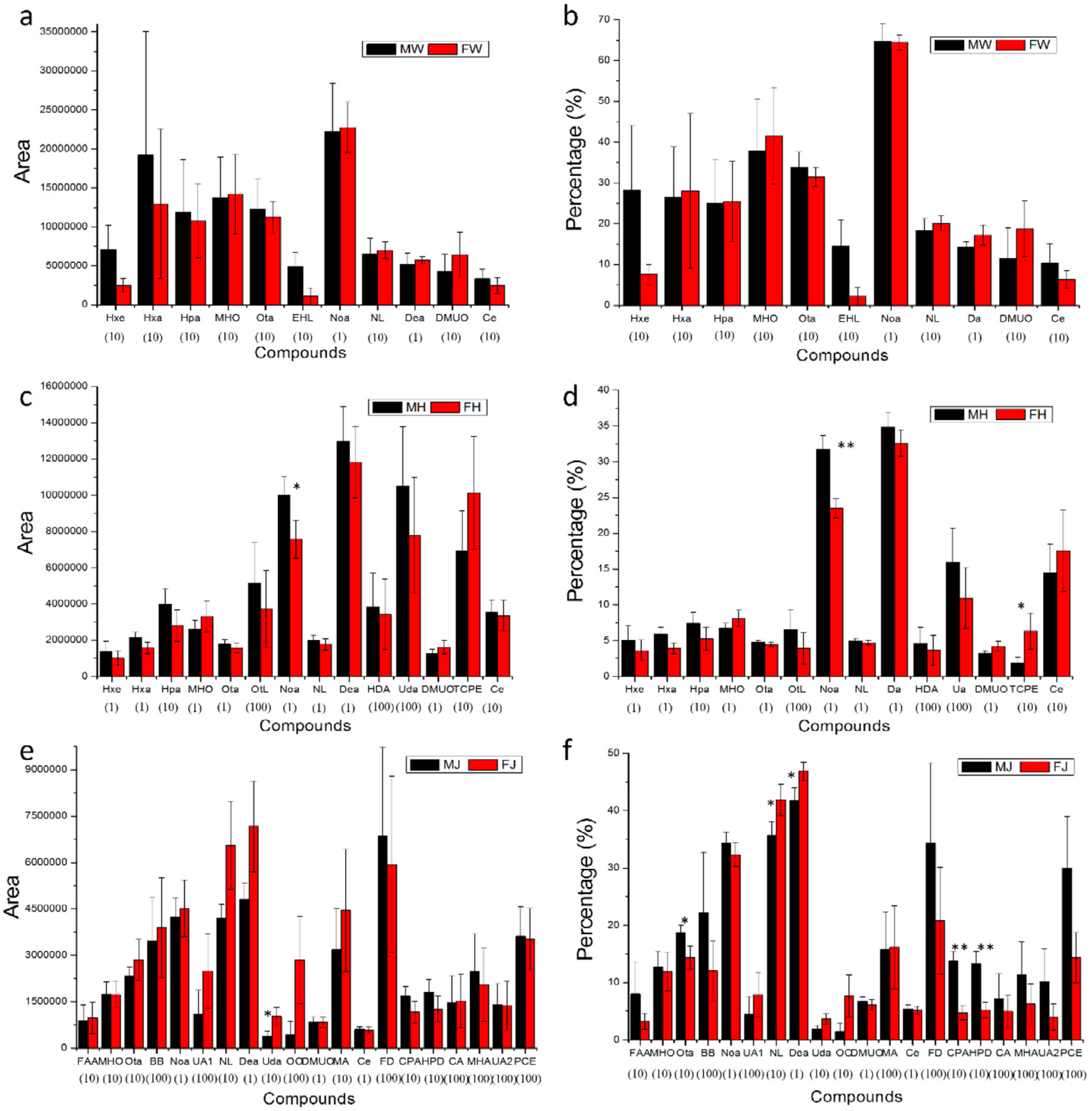

Figure 4. Sex difference in concentration of VOCs emitted from unhatched chicken eggs (W, H, J). (Mean ± SE; M: male; F: female). Asterisk *: 0.05< P < 0.1; **: P < 0.05 above each bar indicates significant difference (P < 0.05/0.01) ($N_{WM} = 5$, $N_{WF} = 5$; $N_{HM} = 35$, $N_{HF} = 29$; N_{JM}=26, $N_{JF} = 26$). (Hxe: Hexane, Hea: Hexanal, Hpa: Heptanal, MHO: 6-methyl-5-Hepten-2-one, Ota: Octanal, EHL: 2-ethyl-1-Hexanol, Noa: Nonanal, NL: 2-Nonen-1-ol, Dea: Decanal, DMUO: 6,10-dimethyl-5,9-Undecadien-2-one, Ce: Cedrene, OtL: 1-Octanol, HDA: Heptadecanamine, Uda: Undecanal, DMUO: 6,10-dimethyl-5,9-Undecadien-2-one, TCPE: Pentanoic acid,2,2,4-trimethyl-3-carboxyisopropyl,isobutyl ester, FAA: 2-fluoro-Acetamide, BB: butyl-Benzene, UA1: Unknown amines-1, OO:9-oxabicyclo[6.1.0]nonan-4-One, MA: N-methyl-1,3-Propanediamine, FD: 1-fluoro-Dodecane, CPA: Cyclopropanecarboxamide, HPD: Heptadecane, CA: Cyclopropanecarboxamide, MHA: 5-methyl-2-Hexanamine, UA2:Unknown amines-2, PCE: Phthalic acid,4-cyanophenyl nonyl ester).

absolute abundance (area) of undecanal (0.05 < P < 0.1) from female J eggs was higher than that from male J eggs.

Meanwhile, average concentrations of hexane emitted from male eggs was found higher than that from female eggs both for W and H breed; mean concentrations of 6-methyl-5-hepten-2-one, 6,10-dimethyl-5,9-undecadien-2-one and cedrene, octanal emitted from female eggs were higher and lower than that from male eggs for all 3 breed, respectively (Figure S4).

Discriminant and Correlation Analysis As might be expected, VOCs emitted from unhatched male and female eggs for W, H, and J breed were well separated in CD model, the accuracy of egg sexing were almost 90% (90−100%), except for 68.8% (area)-76.6% (Percentage) of H eggs (Table S3) and VOCs of female eggs were all trend to the upside of the male eggs (Figure 5 and Table S3). So it is verified that there were some difference between unhatched male and female eggs for 3 breeds. Hexane, 2-ethyl-1-hexanol, 2-nonen-1-ol, decanal, cedrene, etc., nonanal, hexanal, pentanoic acid, 2,2,4-trimethyl-3-carboxyisopropyl, isobutyl ester, etc., decanal, undecanal, cedrene, cyclopropanecarboxamide, etc. mostly contributed to differentiate unhatched (E_0) male and female eggs for W, H, and J breed, respectively. Moreover, nonanal, decanal, 2-nonen-1-ol may contribute greater on the distinction of unhatched male and female eggs and cedrene may contributed greater for W and J eggs (Table S4).

Table 3. Mean levels of VOCs between unhatched male and female eggs for W, H, and J breeds (mean ± SE).

			Peak area		Relative percentage	
W	RT (min)	Volatile compounds	M (5)	F (5)	M (5)	F (5)
	2.67	n-Hexane	707,289 ± 314,503	248,720 ± 86,168	2.84 ± 1.56	0.76 ± 0.25
	7.50	Hexanal	1,919,745 ± 1,586,849	1,293,390 ± 959,108	2.66 ± 1.24	2.81 ± 1.90
	12.56	Heptanal	1,191,947 ± 670,059	1,073,318 ± 471,558	2.51 ± 1.06	2.55 ± 1.10
	17.31	6-methyl-5-Hepten-2-one	1,376,781 ± 521,867	1,418,052 ± 511,202	3.79 ± 1.26	4.15 ± 1.19
	18.08	Octanal	1,225,262 ± 395,234	1,127,781 ± 201,276	3.39 ± 0.37	3.14 ± 0.24
	19.59	2-ethyl-1-Hexanol	491,138 ± 177,650	108,328 ± 108,328	1.45 ± 0.64	1.09 ± 0.00
	23.37	Nonanal	22,245,370 ± 6,172,755	2,2751,195 ± 326,1170	64.77 ± 4.16	64.42 ± 2.12
	26.63	2-Nonen-1-ol	655662 ± 195,328	699,271 ± 107,457	1.85 ± 0.28	2.01 ± 0.21
	28.35	Decanal	5,131,160 ± 1,492,170	5,695,401 ± 463,820	14.37 ± 1.18	17.21 ± 2.76
	39.52	6,10-dimethyl-5,9-Undecadien-2-one	424,690 ± 223,201	640,914 ± 290,067	1.16 ± 0.75	2.35 ± 0.65
	43.83	Cedrene	330,705 ± 131,457	245,510 ± 101,300	1.04 ± 0.48	0.64 ± 0.24
H	RT (min)	**Volatile compound**	**M (35)**	**F (29)**	**M (35)**	**F (29)**
	2.82	n-Hexane	1,344,555 ± 577,628	999,085 ± 394,616	5.01 ± 2.1	3.61 ± 1.48
	7.60	Hexanal	2,138,657 ± 3,00,294	1,571,211 ± 311,178	5.87 ± 0.95	3.95 ± 0.74
	12.62	Heptanal	398,762 ± 86,116	280,835 ± 85,806	0.74 ± 0.15	0.53 ± 0.16
	17.33	6-methyl-5-Hepten-2-one	2,628,461 ± 450,410	3,304,721 ± 834,025	6.76 ± 0.67	8.09 ± 1.16
	18.11	Octanal	1,783,573 ± 242,790	1,568,451 ± 247,404	4.82 ± 0.24	4.43 ± 0.32
	21.57	1-Octanol	51,470 ± 22,393	37,121 ± 21,386	0.07 ± 0.03	0.04 ± 0.02
	23.39	**Nonanal**	**10,018,213 ± 1,002,809**	**7,567,484 ± 1,039,759**	***31.74 ± 1.90***	***23.5 ± 1.33***
	26.64	2-Nonen-1-ol	1,967,318 ± 320745	1,771,096 ± 318,926	4.96 ± 0.36	4.69 ± 0.31
	28.38	Decanal	12,962,365 ± 1,939,274	11,827,630 ± 1,964,846	34.83 ± 2.06	32.58 ± 1.85
	31.72	1-Heptadecanamine	38,494 ± 18,716	34,333 ± 19,362	0.05 ± 0.02	0.04 ± 0.02
	33.05	Undecanal	104,955 ± 32,775	77,739 ± 31,926	0.16 ± 0.05	0.11 ± 0.04
	39.53	6,10dimethyl-5,9-Undecadien-2-one	1,271,976 ± 233,460	1,617,635 ± 365,640	**3.23 ± 0.34**	**4.21 ± 0.65**
	43.79	Pentanoic acid, 2,2,4-trimethyl-3-carboxyisopropyl,isobutyl ester	69,292 ± 22,000	101,251 ± 31,307	**0.19 ± 0.08**	**0.63 ± 0.25**
	43.85	Cedrene	353,624 ± 66,068	334,235 ± 85,153	1.45 ± 0.4	1.76 ± 0.57
J	RT(min)	**Volatile compound**	**M (26)**	**F (26)**	**M (26)**	**F (26)**
	4.32	2-fluoro-Acetamide	89,612 ± 49,903	97,020 ± 50,797	0.80 ± 0.55	0.32 ± 0.14
	17.31	6-methyl-5-Hepten-2-one	174,959 ± 38,928	172,731 ± 42,658	1.27 ± 0.28	1.19 ± 0.33
	18.10	Octanal	232,916 ± 27,542	284,891 ± 66,766	**1.87 ± 0.13**	**1.44 ± 0.20**
	20.73	butyl-Benzene	34,630 ± 14,274	38,886 ± 16,178	0.22 ± 0.11	0.12 ± 0.05
	23.40	Nonanal	4,242,659 ± 609,424	4,521,948 ± 91,3521	34.36 ± 1.85	32.34 ± 2.07
	26.27	Unknown amines-1	11,027 ± 7,670	24,854 ± 12,116	0.04 ± 0.03	0.08 ± 0.04
	26.64	2-Nonen-1-ol	420,446 ± 44,638	656,148 ± 142,147	**3.57 ± 0.24**	**4.19 ± 0.28**
	28.37	Decanal	4,810,998 ± 516,709	7,162,462 ± 14,65934	**41.73 ± 2.31**	**46.91 ± 1.61**
	33.03	Undecanal	**39,338 ± 15,266**	**102,362 ± 29,554**	0.18 ± 0.07	0.36 ± 0.09
	37.77	9-Oxabicyclo[6.1.0]nonan-4-one	4,334 ± 4,334	28,449 ± 14,076	0.01 ± 0.01	0.08 ± 0.04
	39.52	6,10-dimethyl-5,9-Undecadien-2-one	845,923 ± 169,633	829,030 ± 17,3031	6.72 ± 0.77	6.13 ± 0.95
	41.10	N-methyl-1,3-Propanediamine	31,791 ± 13,382	44,538 ± 19,718	0.16 ± 0.07	0.16 ± 0.07
	43.84	Cedrene	602,349 ± 82,750	576,509 ± 101,946	5.42 ± 0.66	5.19 ± 0.69
	44.97	1-fluoro-Dodecane,	68,623 ± 28,688	59,430 ± 28,449	0.34 ± 0.14	0.21 ± 0.09
	46.08	Cyclopropanecarboxamide	167,133 ± 31,618	116,582 ± 35,191	***1.37 ± 0.18***	***0.48 ± 0.12***
	46.22	Heptadecane	180,208 ± 40,670	125,787 ± 41,306	***1.32 ± 0.22***	***0.52 ± 0.14***
	48.08	2-cyano-Acetamide	14,744 ± 8,737	15,242 ± 8,606	0.07 ± 0.04	0.05 ± 0.03
	48.24	5-methyl-2-Hexanamine	247,63 ± 12,251	20,491 ± 11,816	0.11 ± 0.06	0.06 ± 0.04
	48.83	Unknown amines-2	14,114 ± 6,781	13,787 ± 7,863	0.10 ± 0.06	0.04 ± 0.02
	49.38	Phthalic acid, 4-cyanophenyl nonyl ester	36,206 ± 9,541	35,250 ± 10,052	0.30 ± 0.09	0.14 ± 0.04

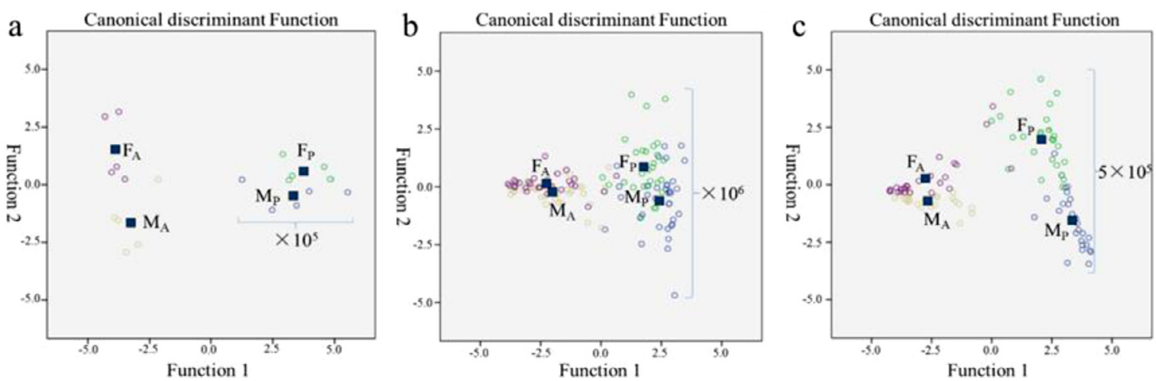

Figure 5. Scatter plots of VOCs emitted from unhatched male and female W, H and J eggs by canonical discriminant analysis. (A: W, B: H, C: J; A (left): area, P (right): percentage; $10^{5/6}$ = magnification of VOC percentage).

Table 4. Correlation between sex and VOCs emitted from unhatched chicken eggs (W, H, J).

	Breeds	**W** (M = 5; F = 5)				**H** (M = 35; F = 29)				**J** (M = 26; F = 26)			
		Area		Percentage		Area		Percentage		Area		Percentage	
RT min	Volatile compounds	R	p	R	p	R	p	R	p	R	p	R	p
2.67	n-Hexane	−0.453	0.189	−0.453	0.189	0.132	0.297	0.140	0.270				
4.32	2-fluoro-Acetamide									0.086	0.546	0.078	0.581
7.50	Hexanal	−0.104	0.774	−0.104	0.774	−0.167	0.187	−0.183	0.149				
120.56	Heptanal	0.070	0.848	−0.070	0.848	−0.132	0.299	−0.146	0.251				
170.31	6-methyl-5-Hepten-2-one	0.104	0.774	0.105	0.773	−0.009	0.942	0.120	0.345	−0.016	0.909	−0.068	0.632
180.10	Octanal	0.174	0.631	−0.035	0.924	−0.076	0.548	**−0.212**	**0.092**	−0.062	0.664	−0.150	0.287
19.59	2-ethyl-1-Hexanol	**−0.557**	**0.094**	***−0.631***	***0.050***					.			
20.73	butyl-Benzene									0.020	0.885	−0.035	0.803
21.57	1-Octanol					−0.061	0.634	−0.075	0.554				
23.40	Nonanal	0.313	0.378	−0.174	0.631	**−0.223**	**0.076**	***−0.385***	***0.002***	−0.115	0.416	−0.094	0.510
26.27	Unknown amine-1									0.120	0.396	0.115	0.415
26.64	2-Nonen-1-ol	0.035	0.924	0.174	0.631	−0.009	0.946	−0.121	0.339	0.027	0.850	***0.279***	***0.045***
28.37	Decanal	0.313	0.378	0.244	0.497	−0.033	0.795	−0.133	0.293	0.005	0.971	0.217	0.123
31.72	1-Heptadecanamine					−0.020	0.873	−0.030	0.815				
33.03	Undecanal					−0.072	0.571	−0.081	0.523	0.221	0.115	0.215	0.126
37.77	9-Oxabicyclo[6.1.0]nonan-4-one									0.200	0.154	0.205	0.144
39.52	6,10-dimethyl-5,9-Undecadien-2-one	0.176	0.626	0.176	0.626	0.055	0.664	0.159	0.209	−0.041	0.773	−0.145	0.306
41.10	N-methyl-1,3-Propanediamine									0.017	0.903	−0.003	0.981
43.79	2,2,4-trimethyl-3-carboxyiso-propyl, Pentanoic acid, isobutyl ester					0.123	0.333	**0.228**	**0.070**				
43.84	Cedrene	−0.349	0.323	−0.140	0.700	−0.052	0.683	−0.063	0.622	−0.123	0.385	−0.083	0.557
44.97	1-fluoro-Dodecane									−0.089	0.533	−0.095	0.501
46.08	Cyclopropanecarboxamide									**−0.238**	**0.089**	***−0.508***	***0.000***
46.22	Heptadecane									−0.207	0.142	***−0.393***	***0.004***
48.08	2-cyano-Acetamide									0.002	0.987	−0.012	0.935
48.24	5-methyl-2-Hexanamine									−0.052	0.715	−0.069	0.626
48.83	Unknown amines-2									−0.039	0.784	−0.069	0.626
49.38	Phthalic acid, 4-cyanophenyl nonyl ester									−0.006	0.967	−0.122	0.390

R: Spearman's correlation coefficient, p: significance value.
Bold: $0.05 < P < 0.1$; bold and italic: $P < 0.05$.

Furthermore, spearman's correlation was used to assess the intrinsic connection between VOCs emitted from unhatched eggs with their sex. Two-ethyl-1-hexanol (area, $0.05 < P < 0.1$; percentage, $P = 0.05$) were found significantly negatively correlated with gender of unhatched W eggs. Octanal (percentage, $0.05 < P < 0.1$), nonanal (area, $0.05 < P < 0.1$; percentage, $P < 0.01$) and 2,2,4-trimethyl-3-carboxyisopropyl pentanoic acid, isobutyl ester (percentage, $0.05 < P < 0.1$) were found significantly negatively and positively correlated with sex of unhatched H eggs, respectively. 2-Nonen-1-ol (percentage, $0.05 < P < 0.1$) and cyclopropanecarboxamide (area, $0.05 < P < 0.1$; percentage, $P < 0.01$), heptadecane (percentage, $0.05 < P < 0.1$) were found significantly positively and negatively correlated with sex of unhatched J eggs (Table 4). Heptadecane has been reported as sex pheromones in 3 species of female moths (Wakamura et al., 2001; Minaeimoghadam et al., 2017).

What's more, hexanal and 6,10-dimethyl-5,9-undecadien-2-one were found to be negatively and positively correlated with sex for W and H eggs, while nonanal was negatively correlated with sex for H and J eggs. More importantly, cedrene was found to be negatively correlated with eggs sex for all breeds, namely, the concentrations of which from unhatched male eggs were higher than female eggs for W, H, and J breed. The relation between most of these sex-related VOCs with sex has been discussed in detail during early of incubation and we won't reiterate it here. It should be stressed that, however, sex-related VOCs emitted from unhatched eggs may be mainly due to differential maternal allocation of resoures other than differential metabolism of embryo, such as estradiol, dihydrotestosterone and so on (Petrie et al., 2001; Kölliker et al., 2012). Fortunately

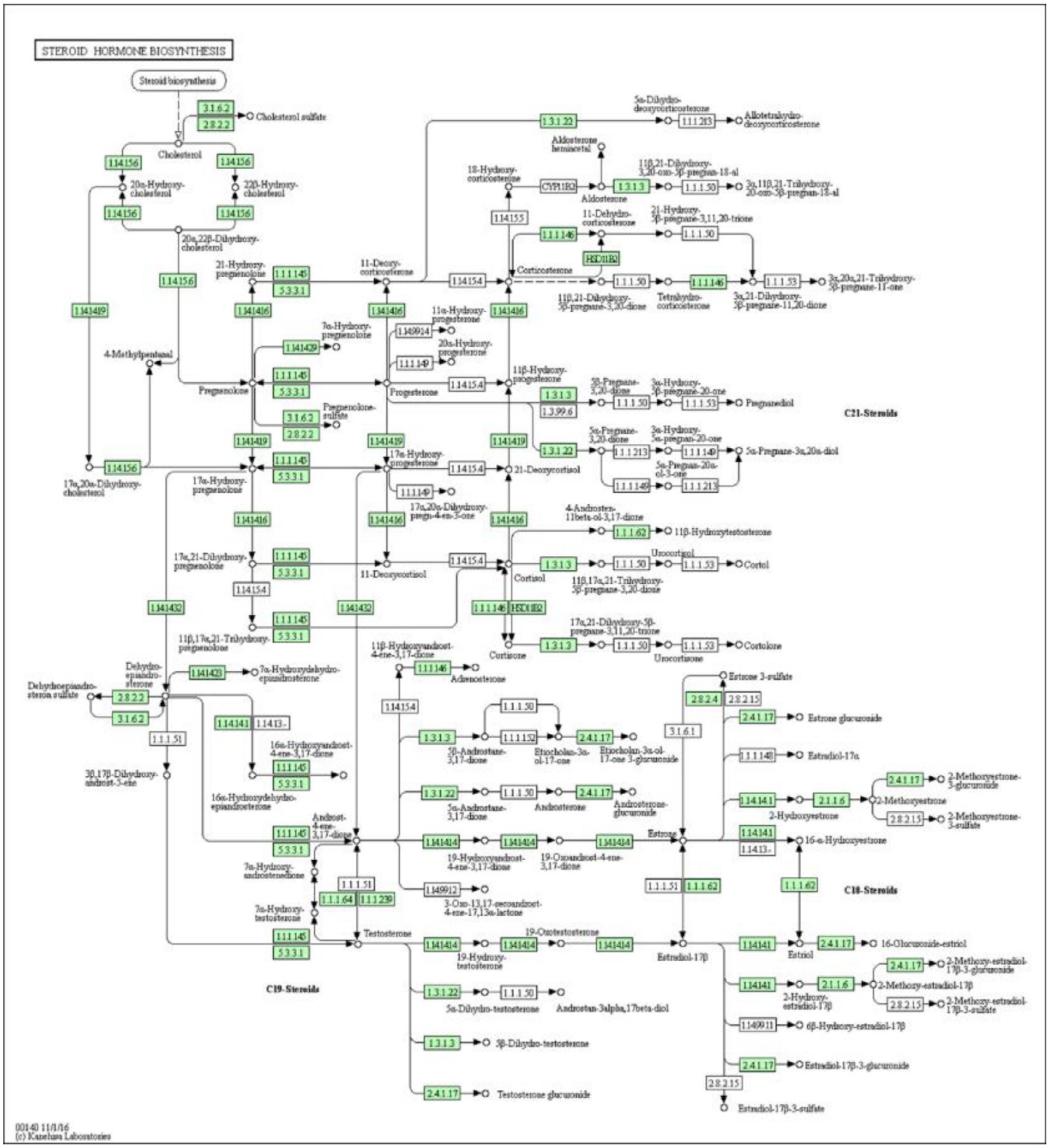

Figure 6. Pathway of sex-related VOCs emitted from unhatched fertilized eggs in KEGG.

and coincidentally, sex-specific VOCs emitted from chicken eggs were found to be related with steroid hormone biosynthesis in KEGG by enrichment analysis using Metabo Anlyst 4.0 (Figure 6).

CONCLUSIONS

Difference in the composition (or content) of VOCs between (hatched) male and female chicken eggs (W) during E_0-E_9 and between unhatched male and female chicken eggs (W, H, and J) were confirmed in this research for the first time. Sex-specific VOCs were strongly influenced by incubation process and egg breed and have been found related with steroid hormone biosynthesis in KEGG. These results will be helpful for understanding the mechanisms of sex identity by cell-autonomous and maternal sex allocation in chicken eggs. More importantly, this study provide a new potential to identify the gender of unhatched fertilized chicken eggs by nondestructive way, although we have neglected the ecological roles of odor emitted from chicken eggs for a long time. Therefore, further works are necessary to investigate the formation mechanism of sex-related VOCs and to put it into practice.

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Informed consent: Informed consent is not applicable for this study.

DISCLOSURES

The authors declare that they have no conflict of interest.

SUPPLEMENTARY MATERIALS

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