ENVIRONMENT, WELL-BEING, AND BEHAVIOR

Fly emergence from manure of Japanese quail fed thymol- or isoeugenol-supplemented diets

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ABSTRACT Many problems in poultry production are caused by a combination of interrelated factors such as management, stress, nutrition, and exposure to pathogens. Saprophagous flies that develop in poultry manure are a potential route of pathogen transmission. Besides being a nuisance, defecation and regurgitation of flies soil equipment and structures and can reduce light levels of lighting fixtures. These effects clearly affect management and may contribute to reductions in poultry egg production, health, and welfare. Many essential oils or their main components have bioactive effects such as natural repellents and insecticides, antioxidants, anticholesterolemics, and antimicrobials. This study evaluated if supplementing quail feed with thymol or isoeugenol as functional food could alter the production of flies from manure. Dropping samples deposited by quail fed with a supplementation of 2,000 mg of thymol or isoeugenol per kg of feed or no supplement (control) were collected. Each sample was incubated inside an emergence cage that was inspected daily to collect emerging adult flies. Fewer flies emerged from droppings of quail fed a thymol-supplemented diet ($P = 0.01$) and there was a tendency to a lower emergence from droppings of isoeugenol-fed quail ($P = 0.09$). The number of positive containers for *Musca domestica* was smaller from quail droppings of thymol- ($P = 0.02$) or isoeugenol- ($P = 0.01$) supplemented feed than from the control counterparts, suggesting an oviposition repellent effect. Supplementing quail feed with thymol or isoeugenol has an overall moderate effect against flies, reducing *M. domestica* emergence.

Key words: dietary supplementation, essential oil, fly control

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INTRODUCTION

Saprophagous flies are mechanical vectors of pathogenic bacteria such as species of *Bacillus*, *Staphylococcus*, *Klebsiella*, *Streptococcus* (Nazni et al., 2005) and enteropathogens such as *Escherichia coli*, and *Aeromonas caviae* (Barnard, 2003); protozoan parasites of humans *Blastocystis hominis*, *Giardia lamblia*, *Cryptosporidium*, and *Cyclospora cayetanensis* (Cárdenas and Martínez, 2004); helminths (de Oliveira et al., 2002); potentially viruses such as avian influenza H5N1 virus (Wanaratana et al., 2011), and Newcastle disease (Barin et al., 2010). In poultry, flies have also been related to salmonellosis (Mian et al., 2002), campylobacteriosis (Ekdahl et al., 2005) and were considered a potential route of transmission of pathogens to newly laid eggs (Axtell and Arends, 1990). Their role as mechanical vectors may be directly related to their abundance (Barnard, 2003), and in large numbers, these insects are a nuisance. In poultry farms, the defecation and regurgitation of flies soils equipment and structures and also can reduce light levels of lighting fixtures. All these effects may contribute to reductions in poultry production and welfare (through incorrect lighting, stress, disease of animals, and so on) as well as decreasing the quality of the eggs (from pollution; Olanrewaju et al., 2010). Flies can also spread to neighboring homes and businesses, and even more, in some regions the overproduction of flies in poultry breaches laws and local public health regulations (Axtell and Arends, 1990).

Most problems in poultry production are caused by a combination of interrelated factors such as handling,

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stress, nutrition, and exposure to pathogens. Stress, for example, can have deleterious effects on animal welfare, productivity, and product quality (Siegel, 1995; Jones, 1996). Because exposure to stressors is inevitable in current management practices, various solutions have been proposed to reduce its negative effects, including direct administration of drugs (Marin et al., 1997; Siegel et al., 2006) and supplementation with functional food (Lábaque et al., 2013). The latter are products that add beneficial properties to the health of those who consume them without having a nutritious value or being included in the vitamin group (Zygadlo and Juliani, 2000). Some essential oils (**EO**) and their main components may be considered functional foods because of their properties such as modulators of gamma-aminobutyric acid activity and fear reducers (anti-stress; Perillo et al., 1999; García et al., 2008; Lábaque et al., 2013), antioxidants (Botsoglou et al., 2002; Luna et al., 2010), anti-cholesterolemics (Crowell, 1999), antimicrobians (Lambert et al., 2001), and antifungical (Adam et al., 1998), among others. The bioactivities of the EO mainly depend on their components, which may vary even within the same plant species (Zygadlo and Juliani, 2000).

Changing the birds' diet could have an effect, in turn, on other health and economic problems associated with poultry production such as flies dwelling in waste (manure, rearing bedding, and so on). The EO, their components, or both may be excreted as bio-transformed products (Hashidoko, 2005) as a result of their passage through the digestive tract. Thus, the droppings of the birds that were fed with these supplements could foster a change in the bacterial microflora that normally develops in the bedding, as many of these compounds have shown antimicrobial effects (Zygadlo and Juliani, 2000), also changing the profile of volatile components. Because the development of fly larvae depend on a relatively small number of bacterial species or metabolic interactions within these microbial communities (Zurek et al., 2000), supplementation of the diet with EO could affect fly productivity indirectly, altering the bacteria growing on the substrate and on which flies depend for their larval development.

Many EO or their main components are effective natural repellents and insecticides against dipterans in the adult and larval stages (Gleiser and Zygadlo, 2007, 2009; Gillij et al., 2008; Palacios et al., 2009a,b; Gleiser et al., 2011). Among the most relevant properties detected against arthropods in general are larvicide, adulticide, oviposition inhibition and repellent (for examples of fly studies, see Sukontason et al., 2004; Bisseleua et al., 2008; Pavela, 2008; Palacios et al., 2009a,b; Tarelli et al., 2009; Kumar et al., 2013). Therefore, fly productivity from manure of EO-supplemented birds could also be affected directly by the presence of these products, for example, creating a toxic environment for the larvae, or an oviposition repellent substrate. The EO components thymol and isoeugenol have fungicidal (Klarić et al., 2007) and bactericidal activity (Lambert et al., 2001; Nostro et al., 2004), and toxicity against arthropods (Table 1).

The reduction of the breeding potential for fly production is of sanitary, economic, and welfare interest, because of these insects' role as pathogen vectors (Nazni et al., 2005), the nuisance activity, and the damage they can cause to the poultry industry. To the best of our knowledge, there have been no previous studies on the influence of modifying diets of birds with functional food on the productivity and diversity of flies that develop in their droppings. This work was designed to assess whether fly production in waste from birds fed a diet supplemented with the components of EO thymol or isoeugenol is reduced compared with fly production in poultry waste from birds fed a conventional diet. The data presented herein are part of a larger project that comprehensively evaluates the effects of adding EO major components as dietary supplements of poultry, with the ultimate goal of creating the foundations for the development of functional food to improve the health and welfare of birds during intensive rearing as well as their productivity and product quality. We used Japanese quail because it is an important agricultural species for meat and egg production in many countries (Baumgartner, 1994) and also a commonly reared species in small home backyard production (Minvielle, 2004). With caution, quail can also be considered a useful model for the extrapolation of data to chickens and other commercially important poultry species (Jones, 1996; Kayang et al., 2006; Poynter et al., 2009).

MATERIALS AND METHODS

The studies were carried out in droppings of Japanese quail (*Coturnix coturnix japonica)* that were kept under standard conditions as described elsewhere (Nazar and Marin, 2011; Luna et al., 2012). Basically, groups of 1 male and 3 females were randomly housed in 1 of 18 cages measuring 20 × 45 × 25 cm (length × width × height) cages. The birds were fed a standard breeder ration (21.5% CP, 2,750 kcal of ME/kg), with feed and water provided ad libitum. Birds were subjected to a daily photostimulatory cycle of 14L:10D with a light intensity of approximately 350 lx during the lighted portion of the day and lights-on occurring at 0600 h daily.

Birds in each cage were randomly assigned to 1 of 3 treatments (6 replicates per treatment) that differed in the supplement added to the feed: untreated control, 2,000 mg/kg of thymol, and 2,000 mg/kg of isoeugenol. A 0.5% ethanolic solution of those supplements was mixed weekly with fresh commercial feed. Supplementations with similar doses have produced desired effects on Japanese quail production and welfare-related parameters (Luna et al., 2012; Lábaque et al., 2013).

From each of the 6 cages per treatment replicates (total n = 18), samples of droppings up to 48 h old were collected and 150 g were placed in open plastic

Table 1. Bioactivity against arthropods of the essential oil components thymol and isoeugenol

Arthropod	Bioactivity	Treatment	Effective/assessed quantity[1]
Isoeugenol			
Acari: Pyroglyphidae			
Dermatophagoides farinae[2]	Adulticide	Contact	$LD_{50} = 5.17$ µg/cm^2
	Adulticide	Fumigant	25.5 µg/cm^2
Dermatophagoides pteronyssinus[2]	Adulticide	Contact	$LD_{50} = 1.55$ µg/cm^2
	Adulticide	Fumigant	25.5 µg/cm^2
Insecta			
Culex quinquefasciatus (Diptera: Culicidae)[3]	Larvicide	Immersion in solution	$LD_{50} = 60$ µg/mL
Musca domestica (Diptera: Muscidae)[3]	Adulticide	Topical	$LD_{50} = 336$ µg/fly
Pediculus capitis (Anoplura: Phthiridae)[4]	Female adulticide	Topical	0.5 mg/cm^2, ≥ 300 min
	Ovicide	Topical	5 mg/cm^2, 67% hatch reduction
Sitophilus zeamais (Coleoptera: Curculionidae)[5]	Adulticide	Topical	$LD_{50} = 30.7$ mg/mg insect
Tribolium castaneum (Coleoptera: Tenebrionidae)[5]	Adulticide	Topical	$LD_{50} = 21.6$ mg/mg insect
Thymol			
Acari: Ixodoidea			
Rhipicephalus (*Boophilus*) *microplus*[6,7]	Adulticide and oviposition	Immersion in solution	1.0–2.0%
	Larvicide	Immersion in emulsion	≥0.5%
	Repellent	Immersion in emulsion	≥0.25%
Insecta			
Aedes aegypti (Diptera: Culicidae)[8]	Larvicide	Immersion	Larvae 1 $LC_{50} = 2.7$ to 17.3 Larvae 2 $LC_{50} = 3.1$ to 23.7
Anopheles stephensi (Diptera: Culicidae)[9]	Adulticide	Vapor toxicity	$LD_{50} = 79.51$ mg
	Oviposition deterrent	Choice condition	100 µg/mL
	Hatch Inhibition	Solution	80 µg/mL
	Larvicide	Solution	$LD_{50} = 48.88$ µg/mL
	Repellent	Vapor toxicity	$RD_{50} = 11.63$ mg
Culex quinquefasciatus[3]	Larvicide	Immersion	$LD_{50} = 30$ µg/mL
Musca domestica[3]	Adulticide	Topical	$LD_{50} = 53$ µg/fly
Agriotes obscurus (Coleoptera: Elateridae)[10]	Larvicide	Topical	$LD_{50} = 196$ µg/larvae
		Fumigation	$LC_{50} = 17.1$ µg/cm^3

[1] LC_{50} = 50% lethal concentration; LD_{50} = 50% lethal dose; RD_{50} = dose repelling 50% specimens.
[2] Kim et al., 2003.
[3] Pavela, 2011.
[4] Yang et al., 2003.
[5] Huang et al., 2002.
[6] de Olivera Monteiro et al., 2010.
[7] Novelino et al., 2007.
[8] Waliwitiya et al., 2009.
[9] Pandey et al., 2009.
[10] Waliwitiya et al., 2005.

containers, following the general procedure described by Goulson et al. (1999). Additional ≈70 g aliquots were collected from each cage and dried under vacuum at 60°C to constant weight. Water content was 64.82 ± 1.98% and did not differ ($F_{(2,15)} = 0.80$; $P = 0.47$) between treatments. The containers holding the samples were placed in groups of 3 (one for each treatment) that were randomly distributed in the quail production area and exposed for 24 h to allow flies to oviposit in the samples. Containers were then placed in emergence traps (Figure 1), which were larger opaque containers (500 mL) with ventilation openings covered with fabric and holding sand for larval pupation. Emerging adults flew up toward the light and were trapped in removable plastic bottles that were provided with ventilation. Emerging adults were removed daily, counted, and identified to species using taxonomic keys of McAlpine et al. (1981, 1987). The number of flies emerging from each treatment (by species or category) and species composition were recorded. Samples were monitored during 40 consecutive days, when no more flies emerged from the samples.

Analysis of variance assessed differences in number of flies emerging (total and per species) and among different supplement treatments (thymol, isoeugenol, and control). Differences between proportions of samples positive for the most abundant fly species were assessed by the proportions test (Analytical Software, 2000). A 2-way ANOVA that assessed the potential interaction between the effects of the supplement treatments with the 3 more abundant species (*Ophyra aenescens*, *Musca domestica*, and *Muscina stabulans*) on the number of flies emerging was also evaluated. Analysis of variance assumptions were evaluated, and whenever necessary, data were transformed to ranks (Shirley, 1987) to meet ANOVA assumptions. Fisher test was used for post hoc comparisons. A P-value of <0.05 represented significant differences.

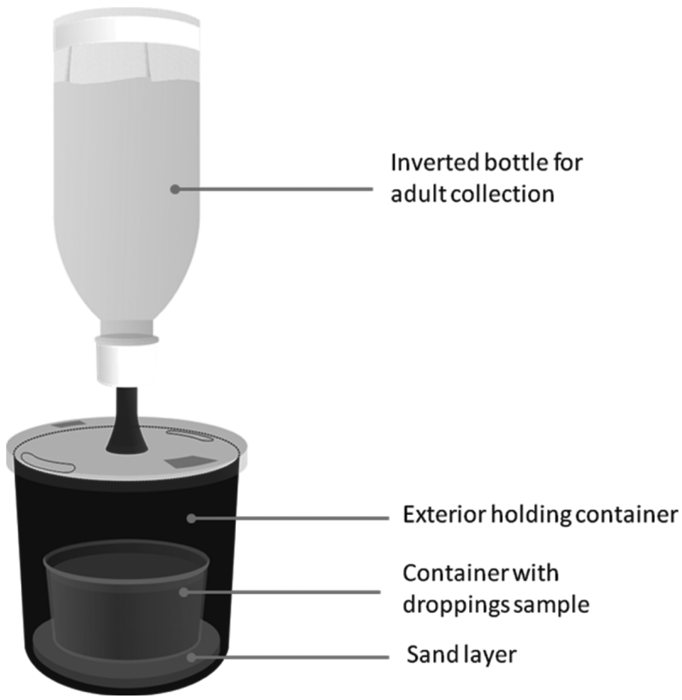

Figure 1. Adult emergence trap where containers holding Japanese quail droppings were placed.

RESULTS

In all, 1,672 flies emerged belonging to 5 families: Calliphoridae (1.3%), Fanniidae (1.9%), Milichiidae (0.1%), Muscidae (92.1%), and Sphaeroceridae (4.7%). The most frequent species were the dump fly *Ophyra aenescens* (34.9%), the house fly *M. domestica* (38.7%), and the false stable fly *Muscina stabulans* (Diptera: Muscidae; 18.5%). One-way ANOVA showed feed supplement effects on total number of flies ($F_{(2,15)} = 4.26$; $P = 0.03$; Table 2) that emerged from quail droppings. The post hoc analysis indicated that fewer flies emerged from droppings of the thymol-supplemented quail compared with control ($P = 0.01$). Comparison of isoeugenol and control samples gives a $P = 0.09$ (Table 2).

Musca domestica emerged from all the control samples and from fewer containers of the isoeugenol (33.3%) and thymol (50%) treatment samples ($P = 0.01$ and $P = 0.02$, respectively). Dump fly and false stable fly emerged from 83.3% of the controls. There were no differences in the percentage of positive containers for dump fly and stable fly between control and isoeugenol ($P = 0.30$ and $P = 0.25$, respectively) or thymol ($P = 0.25$ and $P = 0.30$, respectively) diet droppings.

There were interactions between feed supplements and species for the 3 most frequent fly species ($F_{(4,30)} = 3.33$; $P = 0.02$) on the number of emerging flies (Figure 2). More house flies emerged from the droppings from control supplemented quail compared with the droppings of their isoeugenol- ($P = 0.005$) or thymol- ($P = 0.002$) supplemented counterparts. On the other hand, no differences were detected between control and isoeugenol or thymol in the number of emerging dump flies ($P = 0.42$; $P = 0.26$, respectively) or false stable flies ($P = 0.88$; $P = 0.54$, respectively). In the control droppings, the number of house flies emerging was higher than false stable fly ($P = 0.003$) and dump fly ($P = 0.03$), whereas similar numbers of house fly *M. domestica* and false stable fly emerged from droppings of the isoeugenol- and thymol-treated quail ($P = 0.68$ and $P = 0.44$, respectively). From droppings of birds fed with the isoeugenol supplement emerged almost 4 times more dump flies than false stable flies ($P = 0.05$), and although nonsignificant ($P = 0.12$), more than twice the number of house flies. No differences in the number of these 3 fly species were detected emerging from the thymol-supplemented quail.

DISCUSSION

Waste or by-products of the poultry industry, such as manure and bedding, provide a favorable environment for the development of flies. In neotropical regions, over

Table 2. Flies emerging from manure of Japanese quail fed on standard diet or supplemented with isoeugenol or thymol (2,000 mg per kg of feed)[1]

Taxon	Control	Isoeugenol	Thymol
Calliphoridae			
Lucilia sericata	21 (16.7)	0 (0)	0 (0)
Fanniidae			
Fannia sp.	2 (16.7)	26 (33.3)	4 (33.3)
Milichiidae	0 (0)	0 (0)	1 (16.7)
Muscidae			
Musca domestica	467 (100)	115 (33.3)	66 (50)
Muscina stabulans	85 (83.3)	67 (66.7)	157 (100)
Ophyra aenenscens	207 (83.3)	301 (100)	75 (66.7)
Sphaeroceridae			
Coproica spp.	77 (33.3)	0 (0)	0 (0)
Leptocera spp.	1 (16.7)	0 (0)	0 (0)
Totals	860	509	303
Total fly per sample (mean ± SE; n = 6)	143.3 ± 34.4^{a}	$84.8 \pm 16.1^{a,b}$	50.5 ± 9.9^{b}

[a,b]Total fly number not sharing a common letter show significant differences ($P = 0.01$).

[1]The total number of specimens collected per treatment is shown. The percentage of containers from which each species of fly emerged is indicated in parentheses.

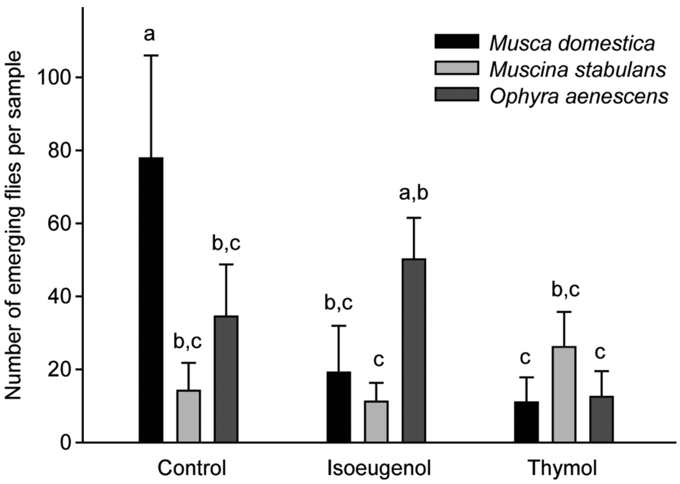

Figure 2. Average number ± SE of *Muscina stabulans*, *Musca domestica*, and *Ophyra aenescens* emerging per container of manure of Japanese quail fed on a standard diet or supplemented with isoeugenol or thymol (2,000 mg per feed kg). Bars with different letters (a–c) differ at $P < 0.05$.

14 species and 12 taxa of Diptera determined to genus or family level have been reported associated with poultry houses (Di Iorio and Turienzo, 2011). In Argentina, studies of flies that affect the poultry industry are concentrated mostly on the control of house fly (Crespo et al., 1998; Lecuona et al., 2007), and according to a recent literature review on insects associated with poultry houses (Di Iorio and Turienzo, 2011), only 3 other species have been reported in this type of environment: *Philornis angustifrons* (Muscidae), *Fannia albitarsis* (Fannidae), and *Hermetia illucens* (Stratiomyidae). Recently, we detected species from the following families developing in Japanese quail droppings: Muscidae, Calliphoridae, Piophilidae, Phoridae, Fanniidae, and Milichiidae (Battán Horenstein et al., 2014). The most abundant species were *M. domestica*, followed by *O. aenescens*, both Muscidae of sanitary relevance (Wanaratana et al., 2011). In the present study we detected 8 different taxa from 5 families of Diptera. As mentioned, flies are not only related to poultry health and welfare problems (Axtell and Arends, 1990; Mian et al., 2002; Ekdahl et al., 2005; Olanrewaju et al., 2010) but also to human health, which is relevant to neighboring homes and to the people directly involved in the birds' husbandry. Among the identified species, *M. domestica* (Barnard, 2003; Nazni et al., 2005) and *M. stabulans* (Patitucci et al., 2010) have been reported as mechanical vectors of bacteria and other parasites.

To the best of our knowledge, there have been no previous studies on the influence of dietary modification on the productivity of flies that develop in the excreta of Japanese quail. The results indicate that dietary supplementation with the essential oil components thymol or isoeugenol modified some parameters of emerging flies. From the droppings of birds supplemented with thymol, fewer total flies emerged than from droppings of birds fed a standard diet. This suggests a toxic or repellent effect (or both) of thymol against flies. The essential oil of thyme (*Thymus vulgaris*), one of whose main components is thymol, have shown toxic effects (topical application) against *M. domestica* (Pavela, 2008) and *Lucilia sericata* (Waliwitiya et al., 2010). Thymol has also proved to be toxic against house fly adults (Pavela, 2011) and has shown genotoxic activity in *Drosophila* (Karpouhtsis et al., 1998). Thymol is also insecticidal against the mosquitoes *Culex quinquefasciatus* (Pavela et al., 2009; Pavela, 2011) and *Aedes aegypti* (Waliwitiya et al., 2009). Moreover, in *L. sericata* it inhibited the flight muscles and there are some indications that thymol acts centrally in blowflies by mimicking or facilitating gamma-aminobutyric acid action (Waliwitiya et al., 2010). Interestingly, this study showed no emergencies of species such as *L. sericata* or *Coproica* spp. from the manure with the feed additives, suggesting that their effects are not limited to the domestic fly.

An inhibition of oviposition has been reported for other Diptera (such as *A. aegypti*; Waliwitiya et al., 2009), and therefore, it is conceivable that there could also be a repellent effect resulting in adult females choosing not to lay their eggs in the substrate containing thymol or its metabolites. This is consistent with fewer positive containers for house flies from thymol-treated samples compared with the controls. However, repellence may differ between species; no differences were detected in the number of positive containers when comparing controls and thymol-supplemented droppings for the false stable fly and dump fly.

Musca domestica larvae feed on decaying material and require an active microbial community for development (Schmidtmann and Martin, 1992; Zurek et al., 2000). Little is known about the contribution of bacteria to the development of fly larvae, and it is unclear whether the larvae need them as a direct source of nutritional supplements (e.g., vitamins), or to metabolize nutrients from natural organic substrates, making them available for nutrition. Probably housefly larvae benefit from complex metabolic interactions within a diverse bacterial community that lead to rapid degradation of organic material, and a large bacterial mass accumulation (Zurek et al., 2000). Regardless of the mode of action, the bacteria are necessary for the larvae to complete their development as they do not do so in sterile media. Because thymol has antibacterial properties (Lambert et al., 2001), it is possible that this compound may have acted indirectly by affecting the larval substrate in which they develop. However, specific studies are needed to confirm this hypothesis.

There are few studies on the effects of isoeugenol on insects, and they show variations in toxicity levels (Khanikor and Bora, 2011). Quail diet supplementation appeared to affect house flies because adults emerged from fewer containers of droppings of isoeugenol-supplemented birds compared with controls. Substrate quality may affect fly development and survival. For example, variations were observed in larval development time, size and weight of pupae, and adult emergence times from different sources of feces, and this was related to moisture and carbon/nitrogen content of the feces (the higher the nitrogen content, the higher the substrate quality; Larrain and Salas, 2008). Considering that bactericidal effects of isoeugenol have been reported against *E. coli* and *Salmonella enterica* (Friedman et al., 2002), it is possible that this component may have an indirect effect on house fly, by directly or indirectly affecting nutrient availability for larvae. On the other hand, no significant differences between treatments were found in the emergence of false stable fly and dump fly from both treated and control quail droppings, appearing to be an adequate nutrient source for the development of these species. Larvae of these species are facultative predators in late stages of their life, and substrate quality for their development would depend on its protein content (Simon et al., 2011). However, it has been shown that there is no predation if eggs or first instar larvae are put together into the larval medium (Farkas and Jantnyik 1990), as was the case in this study (female had access to lay eggs for a short time window). This may be explained by the fact that the house fly *M. domestica* developed faster than the dump fly and false stable fly (on average 13.8 ± 1.0, 17.1 ± 1.0, and 18.4 ± 0.4 d, respectively; $P = 0.001$) and thus only a few or none of its larvae could have been killed by predators. Thus, it is likely that effects of predatory flies, if any, were negligible.

Taken together results suggest that supplementing quail feed with thymol or isoeugenol, at least at the doses assessed, would have a moderate effect on fly production, resulting in an overall reduction of the number of *M. domestica* emerging. The cost of these compounds and other reported positive effects on poultry behavior and meat quality (Luna et al., 2010; Lábaque et al., 2013) support the potential usefulness of these supplements in poultry diets.

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