

Original article (Orijinal araştırma)

Effects of larval crowding on some biological characteristics of the blowfly, *Calliphora vicina* (Robineau-Desvoidy, 1830) (Diptera: Calliphoridae)

Larva yoğunluğunun leş sineği, *Calliphora vicina* (Robineau-Desvoidy, 1830) (Diptera: Calliphoridae)'nın bazı biyolojik özelliklerine etkileri

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Abstract

Blowflies are well known necrophagous insects and usually the first insects to discover and colonize a body after death. Thus, postmortem interval (PMI) can be estimated from the length or stage of development of blowfly larvae collected from a corpse. Abiotic and biotic factors influence multiple traits of a population, including body size, fecundity, survival and development rate. Larval crowding is one of the factors affecting blowfly population dynamics. The purpose of this study was to analyze the effect of larval mass on some life history parameters of *Calliphora vicina* (Robineau-Desvoidy, 1830) (Diptera: Calliphoridae). Experiments were conducted at the Animal Physiology Research Laboratory, Ondokuz Mayıs University during 2017. Five, 25, 50, 100, 500 or 1000 newly hatched *C. vicina* larvae were introduced into a plastic cup containing fresh chicken liver and kept at 22°C and 70% RH under a 12:12 h L:D photoperiod. They were checked at 12-h intervals and development period, survival rate, adult eclosion time, sex ratio, adult size and pupal and adult weights were recorded. The development periods for larval and pupal stages were positively affected by larval crowding. However, larval and pupal survival rate and the percentage of individuals reaching adulthood were very low in the crowded groups. The results also indicated that pupal and adult weight and adult size negatively affected by increasing larval density. It is concluded that larval crowding has an important effect on life history parameters of *C. vicina* and this need to be considered more reliable estimation of PMI.

Keywords: *Calliphora vicina*, larval density, nutrition, survivorship

Öz

Leş sinekleri iyi bilinen nekrofaj böceklerdir ve genellikle ölümden sonra vücudu keşfeden ve kolonize olan ilk böceklerdir. Bu nedenle, ölüm sonrası geçen zaman aralığı (PMI), cesetten toplanan leş sineği larvalarının gelişim evresine veya uzunluğuna bakılarak belirlenebilir. Abiyotik ve biyotik faktörler bir popülasyonun vücut büyüklüğü, verim, hayatta kalma ve gelişim oranı gibi farklı özelliklerini etkiler. Larva yoğunluğu, leş sineklerinin popülasyon dinamiğini etkileyen en önemli faktörlerden biridir. Bu çalışmanın amacı, larva yoğunluğunun *Calliphora vicina* (Robineau-Desvoidy, 1830) (Diptera: Calliphoridae)'nın bazı yaşamsal parametreleri üzerindeki etkisini belirlemektir. Denemeler 2017 yılında Ondokuz Mayıs Üniversitesi, Hayvan Fizyolojisi Araştırma Laboratuvarı'nda yapılmıştır. Yumurtadan yeni çıkan 5, 25, 50, 100, 500 veya 1000 *C. vicina* larvası içerisinde taze tavuk ciğeri bulunan plastik kap içerisine yerleştirilmiş ve 22°C, %70 bağıl nem ve of 12:12 (A:K) s foto periyot koşullarında tutulmuştur. Bu kaplar 12 saat aralıkla kontrol edilerek gelişim süresi, hayatta kalma oranı, ergin çıkış zamanı, eşey oranı, ergin büyüklüğü, pupa ve ergin ağırlıkları kaydedilmiştir. Larva ve pupa dönemlerinin gelişme süreleri larva yoğunluğundan olumlu etkilenmiştir. Buna karşın, larva ve pupaların hayatta kalma oranı ve ergin hale ulaşan bireylerin yüzdeleri kalabalık gruplarda oldukça düşüktür. Sonuçlar ayrıca pupa ve ergin ağırlıkları ile ergin büyüklüğünün larva yoğunluğundaki artıştan olumsuz etkilendiğini göstermiştir. Larva yoğunluğunun *C. vicina*'nın yaşamsal parametreleri üzerinde önemli bir etkiye sahip olduğu ve bu durumun daha güvenilir PMI ölçümü için dikkate alınması gerektiği sonucuna varılmıştır.

Anahtar sözcükler: *Calliphora vicina*, larva yoğunluğu, beslenme, hayatta kalma

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Introduction

Forensic entomology is the scientific study of the use of insects and related arthropods in legal cases including crime scene investigation, abuse and neglect cases, accidents and insect infestation (Amendt et al., 2011). The most important contribution of this science is the estimation of the postmortem interval (PMI), the time between death and the discovery of a corpse (Ireland & Turner, 2006; Verma & Rejant, 2013). Shortly after death, the body begins to decompose and many chemicals released by the body attract different types of insects which lay their eggs on the bodily orifices and open wounds (Brown et al., 2015). Insect colonization of carrion has been demonstrated to occur in a predictable manner called insect succession. Blowflies (Calliphoridae) are usually the first insects to discover and colonize a body (Amendt et al., 2004, 2011).

There are two main methods of using insects to evaluate the elapsed time since death (Catts & Haskell, 1990; Anderson, 1995) using successional waves of insects and based on maggot age and development. The first method is to analyze the predictable, successional colonization of insects on the corpse. Insect succession is used when the victim has been dead for a month or longer. Decomposition is a continuous process and each of these stages is associated with the arrival of different suites of insect species (Amendt et al., 2007; Joseph et al., 2011; Brown et al., 2015; Sharma & Kumar, 2015; Layla et al., 2016). The succession of insect species varies according to the regional climatic conditions and geographical location (Reed, 1958; Payne, 1965). Correct species identification is the major step and knowledge of regional insect succession is required for the success of this method (Joseph et al., 2011).

The second method relies on the determination of the age of the oldest immature insect on the corpse, assuming colonization occurred after death (Catts & Goff, 1992; Joseph et al., 2011). The main entomological approaches to age determination are the use of the species-specific time required for an immature fly to reach developmental landmarks such as length, weight and stages of the life cycle, dependent upon the temperature. By measuring the length or weight of the oldest larva and comparing it with the reference data, the age of the fly larva or maggot may be estimated. Another approach is based on the accumulation of degree hours or degree days that are required for larvae to reach a particular stage of development (Amendt et al., 2004; Ireland & Turner, 2006).

Size and developmental stage of the larvae collected from a body provide a major indication of the PMI. Larval size is affected by factors such as temperature, larval crowding, drugs and quantity of food (Fantinou et al., 2008; Niederegger et al., 2013; Khaliq et al., 2014; Jordan & Tomberlin, 2017).

Vertebrate bodies are an ephemeral and limited nutritional source for insects (Grassberger & Frank, 2004; Shiao & Yeh, 2008) and adult female blowflies may lay eggs on the corpse. The eggs quickly hatch into maggots which consume the corpse. The presence of conspecific individuals may influence the selection of oviposition site by females (Ireland & Turner, 2006; Fantinou et al., 2008; Thiéry et al., 2014). Density-dependent competition for food during the larval stages is considered to be one of the most important factors affecting insect population dynamics (Ireland & Turner, 2006). The competitive feeding environment within more crowded larval cultures resulted in increased or decreased development rates and the production of undersized larvae (Saunders & Bee, 1995).

The larvae of blowflies are used to estimate the minimum PMI stated earlier. Larval length is a factor that can reduce confidence in the accuracy of the estimation of PMI (Weatherbee et al., 2017). Therefore, factors which may affect larval size should be considered to the reliable PMI determinations (Saunders & Bee, 1995; Fantinou et al., 2008; Horváth & Kalinka, 2016; Weatherbee et al., 2017). Larval crowding studies are important for investigating how larval size may lead to inaccurate estimation of the PMI. *Calliphora vicina* (Robineau-Desvoidy, 1830) (Diptera: Calliphoridae) is a Holarctic necrophagous blowfly

species that is found throughout the world (Bonacci et al., 2009). In Turkey, *C. vicina* is the dominant fly species found on a carcass in the winter and autumn (Kökdener & Polat, 2016).

The effects of larval crowding have been investigated with *Calliphora vomitera* (Linnaeus, 1758) (Diptera: Calliphoridae) (Ireland & Turner, 2006) and *Lucilia sericata* (Meigen, 1826) (Diptera: Calliphoridae) larvae (Zheng et al., 2017). To our knowledge, there have been few studies on the effects of larval crowding and competition on *C. vicina*; one such study was performed by Saunders et al. (1999). Separately, the influence of different food substrate on the development of *C. vicina* was determined (Niederegger et al., 2013). Against that background, this study was undertaken to explore the effects of larval overcrowding on some life history parameters of *C. vicina*.

Materials and Methods

A laboratory colony of *C. vicina* was established from adults collected from the campus of Ondokuz Mayıs University, Samsun (41°15' N, 36°19' S), Turkey in 2016. The colony was maintained in gauze covered cages (30 x 30 x 30 cm) at 22°C and 70% RH under a 12:12 h L:D photoperiod. This study was conducted in 2017 at the Animal Physiology Research Laboratory, Ondokuz Mayıs University. Newly emerged adults from the colonies were provided with granulated sugar and water ad libitum. The cages contained a maximum of 300 flies at an approximate 1:1 ratio of males and females (Figure 1). When eggs were required, a plastic beaker containing about 50 g of fresh chicken liver was placed inside a rearing cage and monitored hourly for oviposition. Newly deposited eggs (<1 h old) were transferred into sterile Petri dishes covered with Kimwipes (Eczacıbaşı, Turkey) soaked with deionized water. All Petri dishes were kept in an incubator (Sanyo 36VL) maintained at a constant temperature of 22°C and 70% RH under a 12:12 h L:D photoperiod throughout the experiment. The incubator was monitored hourly and newly hatched larvae were used in the subsequent experiments (Figure 1).

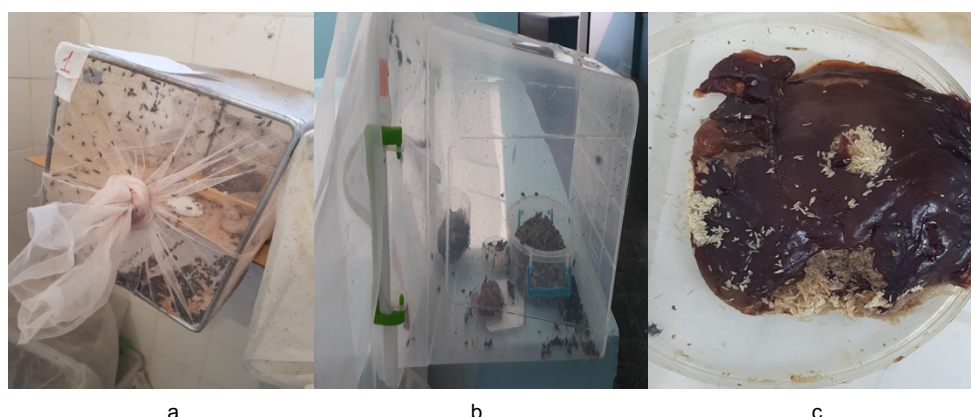


Figure 1. a & b) Rearing cages for *Calliphora vicina*; c) *Calliphora vicina* eggs on liver in petri dishes.

To determine the effects of larval crowding on some life history traits of *C. vicina*, newly hatched larvae were divided into groups at six different densities (5, 25, 50, 100, 500 and 1000 newly hatched larvae). This procedure was repeated four times for each larval density. Post-hatching, the larvae were transferred to a 20 g piece of fresh chicken liver in a plastic cup (15 x 10 x 5 cm). The experimental cups were placed in a larger 500 mL plastic container with a plastic lid with six small air holes and maintained in the incubator at 22°C and 70% RH under a 12:12 h L:D photoperiod. They were checked at 12-h intervals and the development time of larvae in each cup was recorded. As larvae finished feeding and reached the wandering phase, they left the food. At that stage, plastic cups containing the non-feeding larvae and any remaining food were removed from the container and non-feeding larvae were transferred to 1 L glass jars containing about 10 g of dry sawdust for pupation. Jars were sealed with a fine mesh cover and maintained

at 22°C and 70 % RH under a 12:12 h L:D photoperiod for adult emergence. Pupation and eclosion times, pupation success, pupal and adult weight and sex ratio were recorded. Adult flies were collected and killed by freezing at -20°C. They were then removed from the freezer and separated by gender. The size of each adult was measured using a stereomicroscope equipped with a digital camera (Leica MZ 12.5, LAS Version 3.8.0, Leica Microsystems, Switzerland) at a magnification of 10x. Three measurements were taken, namely the length of the posterior cross vein (dm-cu) of the left wing, costa distance between the R₂₊₃ and R₁, and the length of the mesothoracic tibia (Laparie et al., 2016).

Statistical analysis

All statistical analysis was performed with the SPSS software, version 22.0. The effects of population density on the larval and pupal development time and pupal weight of *C. vicina* were subjected to one-way analysis of variance (ANOVA). The Tukey-Kramer HSD test at the level of significance P=0.05 was used to determine the significance of means. Adult weight and adult size differences were analyzed by using two-way ANOVA, with the variables being density and gender. In addition, post-hoc multiple comparisons were performed by using Fisher's least significant difference test.

Results and Discussion

The time required for the development of *C. vicina* larvae and pupae at different levels of larval density are given in Table 1.

Table 1. Development time of *Calliphora vicina* immature life stages at different larval densities

Larval density (n)	Stages (h; mean±SE)	
	Larva	Pupa
5	192.0±0.70 a*	379.0±2.48 a
25	192.6±0.40 a	377.0±4.07 a
50	192.8±0.37 a	374.0±4.59 a
100	168.6±0.40 b	371.0±2.52 b
500	167.8±0.37 b	338.4±2.92 b
1000	160.0±1.14 b	336.0±4.11 b

*Means in the same column followed by the same letter are not significantly different (P=0.05).

The data indicated that increasing larval density led to changes in the larval and pupal development time of *C. vicina*. For their larval development period, the mean values for 100, 500 and 1000 larvae were significantly different (F=570, P=0.0001) and for pupal development time, mean values over a density of 50 were all significantly different (F=21.2, P=0.0001). These results are similar to those obtained by Goodbrod & Goff (1990) for *Chrysomya megacephala* (Fabricius, 1794) (Diptera: Calliphoridae) and *Chrysomya rufifacies* (Macquart, 1843) (Diptera: Calliphoridae). Similarly, Zayed (2004) reported that the duration of development in the immature stages of *Culex pipiens* (Linnaeus, 1758) (Diptera: Culicidae) decreased as larval density increased. The development period for *C. vicina* shortened with increased larval density (Saunders & Bee, 1995). Also, Ireland & Turner (2006) demonstrated that crowded larval cultures reared on liver and muscle had shorter developmental periods than those reared at low densities. In nature, animal cadavers are important food resources and each female blowfly lays many eggs on a corpse. Large numbers of larvae hatch from these eggs, and then feed on the carcass. Larval aggregation is beneficial for feeding larvae because it causes the secretion of proteolytic enzymes and ammonia by larvae (Reis et al., 2001). These secretions macerate the food externally and lead to more efficient feeding for larval mass. Charabidze et al. (2011) and Kotzé et al. (2016) also observed that gregarious feeding

behavior leads to a local temperature increase among blowfly larvae and this situation affect larval development. These previous studies could help to explain the reasons for faster development in the more crowded cultures. Decreased development time is usually accompanied with the reduction of body size (Saunders & Bee, 1995; Zayed, 2004). In the present study, the response of the blowflies to food competition during larval stage is to exhibit plasticity in the range of size, and thus smaller-sized individuals can be produced in pupal and adult stages. These smaller adults can still lay normal-sized and viable eggs, but usually in smaller numbers; however, an otherwise high risk of predation during immature stages is moderated. Contrary to our findings, Al-Misned (2002) reported a significant increase in the larval development time of *Wohlfahrtia nuba* (Wiedemann, 1830) (Diptera: Calliphoridae) at increasing larval densities. Similarly, Manorenjitha & Zairi (2012) showed that overcrowding and starvation of the larvae of the mosquito *Aedes albopictus* (Skuse, 1894) (Diptera: Culicidae) prolonged larval growth up to 36 d. Sokal & Sullivan (1963) also reported that the length of the development period of the immature stages of the house fly increased as larval density increased. Taken together, these data indicate that larval crowding affects the development of immature stages of insects differently.

In the present study, the rearing density of the larvae of *C. vicina* affected the survival rate of immature stages (Table 2). Larval and pupal survival and the percentage of individuals reaching adulthood were very low in the crowded cultures. There was a significant effect of population density on the number of surviving pupa and larvae (for pupae $F=519$, $P=0.002$; for larvae $F=289$, $P=0.001$) and adults ($F=21.7$, $P=0.001$). In a previous study, Saunders & Bee (1995) reported that about 1 g of minced beef muscle is sufficient for the full development of a larva of *C. vicina*. Here, 20 g of fresh chicken liver was used for each group. When the initial larval density is above 25 larvae per 20 g of diet, an important decrease was observed in the survival rates of larvae in our study. This result appears to be consistent with the observations of Saunders & Bee (1995)'s. Thus, intraspecific competition for limited food among larvae may lead to high mortality. In addition, higher larval density may induce more stress and then aggressive contacts increase among larvae, this could increase their mortality. Our findings are consistent with those reported for *C. vomitera*, *C. pipiens*, *Aedes aegypti* (Linnaeus, 1762) (Diptera: Culicidae) and *A. albopictus* (Zayed, 2004; Ireland & Turner, 2006; Arnaldo, 2009; Manorenjitha & Zairi, 2012). Fantinou et al. (2008) showed that larval crowding had a significant effect on larval survival of *Sesamia nonagrioides* (Lefebvre, 1827) (Lepidoptera: Noctuidae). On the contrary, Reigada & Godoy (2006) found that the survival of *C. megacephala* was not affected by larval densities (200 or 1000 larvae) at the same temperature. Likewise, Al-Misned (2002) demonstrated that the percentages of pupal and total survival were not significantly correlated with population density in *W. nuba*.

Table 2. Survival rate of *Calliphora vicina* at different larval densities

Larval density (n)	Survival rate				Eclosion		Adult sex ratio			
	Larvae		Pupa				Female		Male	
	n	%	n	%	n	%	n	%	n	%
5	5	100.0	5	100.0	5	100.0	11	56.0	9	44.0
25	24	96.0	24	96.0	24	96.0	59	61.0	37	29.0
50	30	60.0	30	60.0	25	50.0	56	56.0	44	44.0
100	59	59.0	58	58.0	49	49.0	100	51.0	96	49.0
500	282	56.4	268	53.6	161	32.2	300	47.0	344	53.0
1000	393	39.0	305	30.5	265	26.5	508	48.0	552	52.0

The effects of larval density on the pupal and adult weight of *C. vicina* are presented in Table 3. Statistical analysis revealed a significant difference in pupal weight among different larval densities ($F=31.2$, $df=5$, $P=0.0001$). In addition, both density ($F=325$, $df=5$, $P=0.001$) and sex ($F=16.2$, $df=1$, $P=0.001$) significantly affected the adult weight of *C. vicina*. Similarly, Agnew et al. (2000) found that mosquitoes emerged with lighter starved dry adult weight as larval density increased. Al-Misned (2002) also reported that the increasing larval population density resulted in a decrease of pupal and adult weights of flesh fly, *W. nuba*. It is known that overcrowding during the larval stages of development results in a competitive feeding environment. As more larvae competed for the same amount of food, the larvae may force to metamorphose with an insufficient food reserve, resulting in pupae of reduced weights and these smaller pupae gave rise to smaller adult blowflies.

Table 3. Weights of pupal and adult stages of *Calliphora vicina* at different larval densities

Larval density (n)	Pupal weight (mg; mean±SE)	Adult weight (mg; mean±SE)			
		Female		Male	
5	772±45 a*	737±94 aA**		649±218 aA	
25	750±45 a	261±15 bA		257±99 bA	
50	750±16 a	143±50 cA		134±40 cA	
100	587±92 b	115±33 cdA		109±43 cdB	
500	266±55 c	86±46 deA		78±46 deA	
1000	180±48 d	54±23 eA		53±222 eA	

* Means in the same column followed by the same letter are not significantly different ($P=0.05$).

** The same uppercase letter in the same line indicate that the means are not significantly different.

Analysis of data for the effects of larval population density on the adult size of *C. vicina* is shown in Table 4. This study determined that density ($F=0.253$, $df=5$, $P=0.939$) and sex ($F=0.23$, $df=2$, $P=0.978$) did not significantly affect the mean length of the posterior cross vein (dm-cu) in *C. vicina*. In contrast, for costa distance (between the R_{2+3} and R_1) the effects of density were significant ($F=62.9$, $df=5$, $P=0.001$). However, sex had no effect on the costal distance (between the R_{2+3} and R_1) of adults ($F=0.376$, $df=1$, $P=0.540$). Larval density also significantly affected the length of the tibia ($F=7.86$, $df=5$, $P=0.001$) but no significant difference was observed among females and males ($F=2.89$, $df=1$, $P=0.089$). In a previous study, Saunders & Bee (1995) reported similar results for *C. vicina*. Similarly, Smith & Wall (1997) showed that for both *L. sericata* and *C. vicina*, the size of male and female adults declined with increasing initial larval number. In addition, Zayed (2004) also found that the female wing length of *C. pipiens* increased as the larval density decreased. All these authors have emphasized the importance of increasing levels of exploitative competition for limited resources among larvae.

In conclusion, our results revealed that larval crowding can have a considerable effect on various life history traits of *C. vicina*. The immature development of *C. vicina* accelerated at high densities which could mislead the forensic entomologist during criminal investigation procedures. Separately, survival percentage of the immature stages, pupal and adult weights and the body size of the *C. vicina* was correlated negatively with increasing larval density. This would explain the lower eclosion percentage associated with higher density rearing. Furthermore, larval crowding affects the size of *C. vicina* and development times and causes undersized individuals. These adverse effects of overcrowding were probably due to differences in food availability and the reduced living space of immature stages. There may also be a buildup of toxic metabolic byproducts.

Table 4. The effect of larval population density on adult size of *Calliphora vicina*

Larval density (n)	Sex	Cross vein length (mm; mean±SE)	Costa distance R ₂₊₃ and R ₁ (mm; mean±SE)	Tibia length (mm; mean±SE)
5	M	2582±109 a*	4381±380 a	3360±282 a
	F	2629±94 a	4470±232 a	3549±263 a
25	M	2533±64 a	4279±118 a	3535±113 ab
	F	2559±83 a	4577±122 a	3679±103 ab
50	M	2519±66 a	4137±149 ab	3482±100 ab
	F	2577±61 a	4337±137 ab	3565±86 ab
100	M	2109±42 a	3933±78 b	3400±63 ab
	F	2027±42 a	3962±78 b	3497±65 ab
500	M	1755±27 a	3466±39 c	3119±39 b
	F	1790±29 a	3667±45 c	3158±42 b
1000	M	1669±49 a	3316±32 c	3001±30 b
	F	1555±22 a	3461±34 c	3169±121 b

*Means in the same column followed by different letters are significantly different.

These results highlight the necessity of a better comprehension of the effects of larval mass on PMI estimation in the context of forensic entomology. Some authors when evaluating larval masses do not mention the effect on the accuracy of postmortem interval estimates (Benecke, 1998; Introna et al., 1998). Heaton et al. (2014) stated that the temperature of maggot masses differ significantly from ambient and elevated mass temperatures may influence larval development rates. However, the effects of larval crowding generally on PMI estimation are unclear and much work is still required to untangle the complex relationships and reveal deeper forensic insights. Future experiments with the different size larvae of different species in combination with field studies would be useful to the understanding of variation in PMI estimates.

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