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# Effects of ante-mortem use of methadone on insect succession patterns

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## Abstract

**Background:** Evaluation of insect succession patterns is a scientific method to estimate the time elapsed since death. Several studies have shown that ante-mortem intake of opioids affect maggot growth rate. However, there are few published data that investigate the effect of ante-mortem opioid use on insect succession patterns. Therefore, the main purpose of this research was to investigate the effect of methadone on the succession patterns of insects on rabbit carcasses during the spring and winter of 2019. In the present study, the H-null hypothesis represents the dissimilarity between the successional waves of species from testing and control carcasses (H0: P: 0).

**Results:** During this study, 15 and 13 insect species were collected from carcasses during the spring and winter, respectively. The most dominant species during the both seasons were *Chrysomya albiceps* and *Calliphora vicina*. These two species preferred to lay eggs on the control carcasses earlier than the treated carcasses. *Lucilia cuprina* was observed only from the remains of untreated carcasses, while *Saprinus chalcites* and *Necrobia rufipes* were recorded only from the remains of treated rabbits. Samples indicate that 11.8% of the insects were members of the Coleoptera. Permutation analyzes based on the Mantel test were  $0.647 \pm 0.16$  ( $P = 0.009$ ) and  $0.693 \pm 0.16$  ( $P = 0.003$ ) for the similarity of the species between treated and untreated carcasses in the spring and winter, respectively. Permutation analyzes for the two most dominant fly species (*Ch. albiceps* and *C. vicina*) between the treated and untreated carcasses in the spring and winter were  $0.515 \pm 0.15$  ( $P = 0.05$ ) and  $0.491 \pm 0.14$  ( $P = 0.09$ ), respectively.

**Conclusion:** The results revealed that the overall pattern of insect succession was similar between the treated and untreated rabbit carcasses. However, the patterns of succession of *Chrysomya albiceps* and *Calliphora vicina* differed slightly between treated and untreated carcasses, and this could have an effect on the PMI min estimate.

**Keywords:** Forensic entomology, Insect succession, Methadone

## Background

Synthetic opioid death continues to rise in different parts of the world (Wolff, 2002; Akhgari et al. 2018; Concheiro-Guisan et al. 2018). There are more than 42,000 deaths due to opioid overdose in the United States (Concheiro-Guisan et al. 2018). Methadone is a semisynthetic  $\mu$ -opioid receptor agonist that prescribed for the treatment of opioid addiction (Hsieh et al. 2018).

In a retrospective study of cases from Tehran, Iran, between 2009 to 2015, there were 1274 deaths in patients in whom methadone was detected in the blood (Akhgari et al. 2018).

In a number of drug-related deaths, the corpse is found after a few days or weeks when the decomposition process is in its mid or late stages. In such cases, forensic entomology is a reliable scientific discipline to estimate the time since insect colonization or the minimum post-mortem interval (PMImin) (Tabor et al. 2005). The degree of development and succession patterns of insects are two main techniques for estimating PMI. By the

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successional method, the PMImin of a corpse is estimated by comparing the composition of corpse insect fauna with the composition of baseline fauna (Amenndt et al. 2007). Necrophagous species include dipteran and coleopteran species are the most important orders for PMI estimation in different stages of decomposition (Watson, 2004).

The effects of drug/toxin on body weight and body length as well as the rate of development of insects have been investigated in several, for example; heroin, codeine, and methamphetamine decrease larval development time and increase the larval length (Madison Lee Goff et al. 1991; M Lee Goff et al. 1997; Fathy et al. 2008).

The effect of methadone on the development rate/time of maggots has also previously been investigated and found to be responsible for retardation in the developmental time of *Lucilia sericata* (Hecht et al. 2007), but few published data are available on the effect of methadone on successional pattern.

As a consequence, the objectives of this research are to estimate whether antemortem use of methadone affects the successional patterns of insects on decaying remains and, finally, estimate the species diversity and fauna of the local communities of insects in the study area.

## Materials and methods

### Study sites

The study was conducted during the winter and spring of 2019 at the Kazerun Health Research Station (29°37'10"N 51°39'15"E), located in Kazerun County, southwestern Fars province, Iran. Kazerun is 860 m above sea level and the climate in the region is a subtropical steppe type with an average annual temperature of 21.6 °C and precipitation rate of 257 mm (Keshavarzi et al. 2020).

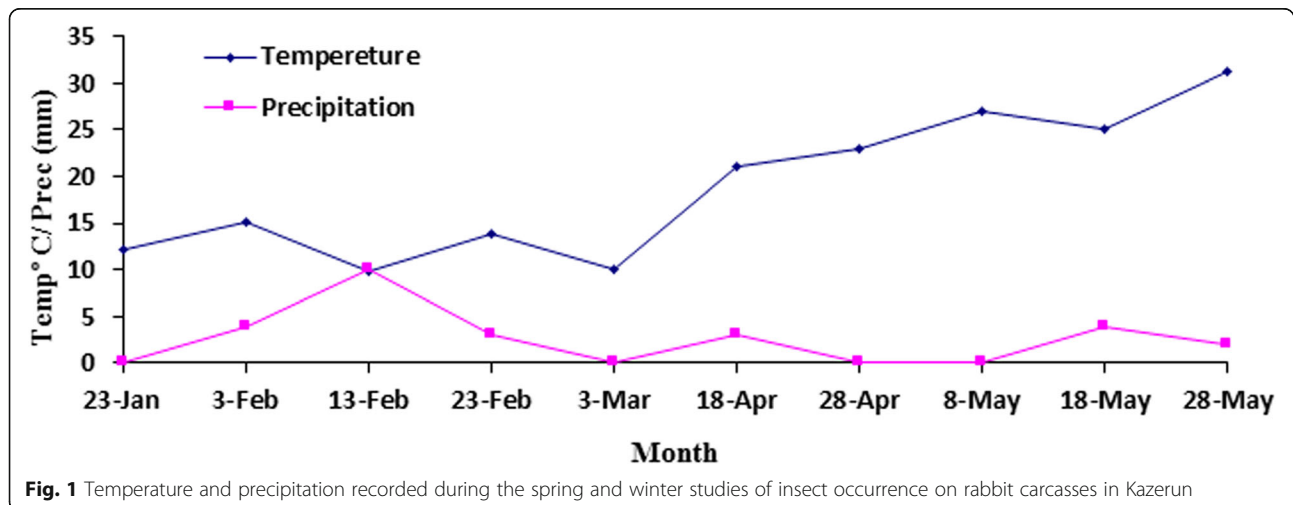
The first trial began on the 23rd of January and was terminated on the 26th of March and the second trial began on the 20th of April, and was terminated on the 23rd of May. Temperature and precipitation data were obtained from the closest meteorological stations and from multiple measurements done on the site (Fig. 1).

### Carcasses and methadone dosing

In the present study, two replicates were performed in each season and four rabbits were used in each replicate. During each trial, four rabbits ( $\approx$  1.8–2 kg) obtained from the Animal Lab at Shiraz University and divided into two groups, including addicted rabbits and non-addicted or control. All of the rabbits were maintained on a 12-h light/dark cycle and unlimited access to food and water. In order to imitate real human methadone use, addicted group was treated with methadone for three consecutive weeks. They received 2 mg/kg/day methadone orally via a gastric tube and then gradually increased the dose to 20 mg/kg/day. At the end of the period, to confirm that rabbits were methadone dependent, Naloxone (0.5 mg/kg I.M.) was injected and opiate withdrawal symptoms (i.e., ptosis and teeth chattering) were observed. At the end of the treatment period, the animals were killed by chloroform in the morning and placed inside separate wire cages (75 × 50 × 65 cm) with 2 cm steel-welded tubing, surrounded with 1.4 cm mesh. Cages were placed directly in contact with the ground in the study area. Permission to use rabbits as the research animal for the present study was granted by the Ethical Committee of Tehran University of Medical Science.

### Protocol for sampling and statistical analysis

Insects samples were collected twice daily at 10 am and 16 pm during fresh and bloat stages of decomposition



and then once per day for the next stages (Tabor et al. 2005). We collected eggs and larvae from different parts of the carcasses with forceps or by hand, and also we used pitfall traps to catch post-feeding maggots. In the present study, immature stages of flies were chosen for the study of succession patterns and during each visit, adult flies activity was recorded to investigate their oviposition behavior. Approximately, 25 larvae were collected from each maggot mass during samplings and then they were put in hot water to die, and another group of 25 larvae was collected for rearing to the mature stage. For the identification of insect samples, different approved taxonomic keys were used (Velásquez et al. 2010; Akbarzadeh et al. 2015; Ghahari et al. 2015; Grzywacz et al. 2017).

Jaccard similarity coefficient was used to determine the species match between sampling intervals within the experimental carcasses. The Jaccard index ranges from 0 to 1 showing complete dissimilarity to perfect match between sampling intervals for any insect species (Tabor et al. 2005; Keshavarzi et al. 2019). Average similarities of species between the sampling intervals were calculated for both treated and control groups. A permutation approach based on a Mantel correlation statistic was used to determine the degree of similarity in the successional patterns of insects.

In the present study, the H-null hypothesis represents the dissimilarity between the successional waves of species from testing and control carcasses ( $H_0: P = 0$ ). The analysis was done with PAST software version 3.14 (Paleontological Statistics Software Package). The accuracy of Jaccard similarity between groups was tested by the Jackknife method ( $P_i = J + (n - 1)(J - J_i)$ ), where  $n$  is the number of sampling intervals,  $J$  is the overall resemblance and  $J_i$  is the partial estimate of  $J$  when it sampling interval is removed (Dixon, 2001).

We used the two measures of Hill numbers of order  $q$ : Shannon index ( $q = 1$ ) and the inverse Simpson index ( $q = 2$ ) to compare the species diversity between the two seasons (KHOODEL et al. 2020). We hypothesized that species diversity metrics will show difference between groups. Hill numbers (or the effective number of species) as a statically method, quantifying the species diversity of a community (Chao et al., 2014b). We used the sample-size based rarefaction and extrapolation (R/E) curves with 95% confidence intervals based on a bootstrap method for comparison of species diversity between the seasons. The analysis was performed with an R package (iNEXT) that provided by Chao et al. (2014a) (Chao et al., 2014b). The detailed formulas and information for the Hill numbers and iNEXT package can be found elsewhere (Chao et al., 2014a; Chao et al., 2014b).

## Results

In this study, 15 and 13 insect species were identified in the spring and winter, respectively. The mean numbers of insects collected in the winter and spring were 621 and 912 individuals, respectively. Succession waves of insects on the remains of untreated and treated rabbits for each sampling day are shown in Tables 1 and 2. Temperature fluctuations were greater in winter than in spring. As temperature increased, the decomposition process occurred faster. When temperature increased in the spring, the activity of some species of Calliphoridae, such as *Calliphora vicina* decreased, but some other species, such as *Chrysomya albiceps* and *Lucilia sericata* increased. In the winter, *C. vicina* activity, unlike other species, was not inhibited in the vicinity of the carcasses on rainy days.

The present study demonstrated five stages of decomposition including, fresh, bloated, decay, post-decay, and dry. The mean duration of the decomposition process was shorter in spring than in winter ( $p = 0.01$ ) (Table 3). The mean duration of the fresh, bloated, and dry stages was the same in the treated and control carcasses. But the duration of the decay and post-decay stages was shorter in untreated carcasses than in treated carcasses.

Twelve dipteran species were found in 3 families in the study. Ten of the dipteran species were identified on both treated and untreated carcasses; however, two of them (*Sarcophaga* spp., *Lucilia cuprina*) were observed only on untreated carcasses. *Chrysomya albiceps* and *C. vicina* were the dominant fly species in the spring and winter, respectively. Dipteran species were the first visitors during the spring and winter. *Chrysomya megacephala* was restricted to the spring on the remains of both treated and untreated rabbits. The most frequent dipteran larvae were found on untreated carcasses, but the most activity of adult flies was observed in the vicinity of the treated carcasses during fresh and bloated stages. Significant difference was not found in the comparison of the abundance of insect species on treated and untreated carcasses in each season by Mann–Whitney  $U$  test ( $U = 103.5$ ,  $P = 0.73$  for spring and  $U = 49.5$ ,  $P = 0.123$  for winter).

Diversity analysis based on Hill numbers for Shannon and Simpson indices showed high species diversity in spring (Fig. 2a, b). Shannon and Simpson indices were significantly different between the two seasons, as their confidence intervals separated.

Coleopterans were shown to be 6 species in 4 families. Four species of beetles have been collected from both treated and untreated carcasses. *Creophilus maxillosus* (Staphylinidae) was the most dominant beetle species comprising > 60% of the beetle specimens collected. *Saprinus chalcites* (Histeridae) and *Necrobia rufipes* (Cleridae) were recorded only from the remains of

**Table 1** Insect\* succession patterns for methadone-treated and untreated rabbit carcasses during 15 sampling intervals in spring 2019

Family	Taxa	Sampling Interval (Days)														
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Calliphoridae (Blow flies)	<i>Calliphora vicina</i>	[Grey bar from day 1 to 15]														
	<i>Chrysomya albiceps</i>	[Black bar from day 1 to 15]														
	<i>Chrysomya megacephala</i>	[Grey bar from day 1 to 15]														
	<i>Lucilia sericata</i>	[Black bar from day 1 to 15]														
Sarcophagidae (Flesh flies)	<i>Sarcophaga aegyptica</i>	[Grey bar from day 1 to 15]														
	<i>Sarcophaga argyrostoma</i>	[Black bar from day 1 to 15]														
	<i>Sarcophaga spp</i>	[Grey bar from day 1 to 15]														
Muscidae (House flies)	<i>Musca domestica</i>	[Grey bar from day 1 to 15]														
	<i>Muscina stabulans</i>	[Black bar from day 1 to 15]														
Histeridae (Clown beetles)	<i>Hister spp</i>	[Grey bar from day 1 to 15]														
	<i>Saprinus chalcites</i>	[Black bar from day 1 to 15]														
Cleridae (Checkered beetles)	<i>Necrobia rufipes</i>	[Grey bar from day 1 to 15]														
Dermestidae (Larder beetles)	<i>Dermestes maculatus</i>	[Black bar from day 1 to 15]														
Staphylinidae (Rove beetles)	<i>Creophilus maxillosus</i>	[Grey bar from day 1 to 15]														
Formicidae (Ants)	<i>Messor spp</i>	[Black bar from day 1 to 15]														

\*Immature stages of flies, adult beetles, and ants

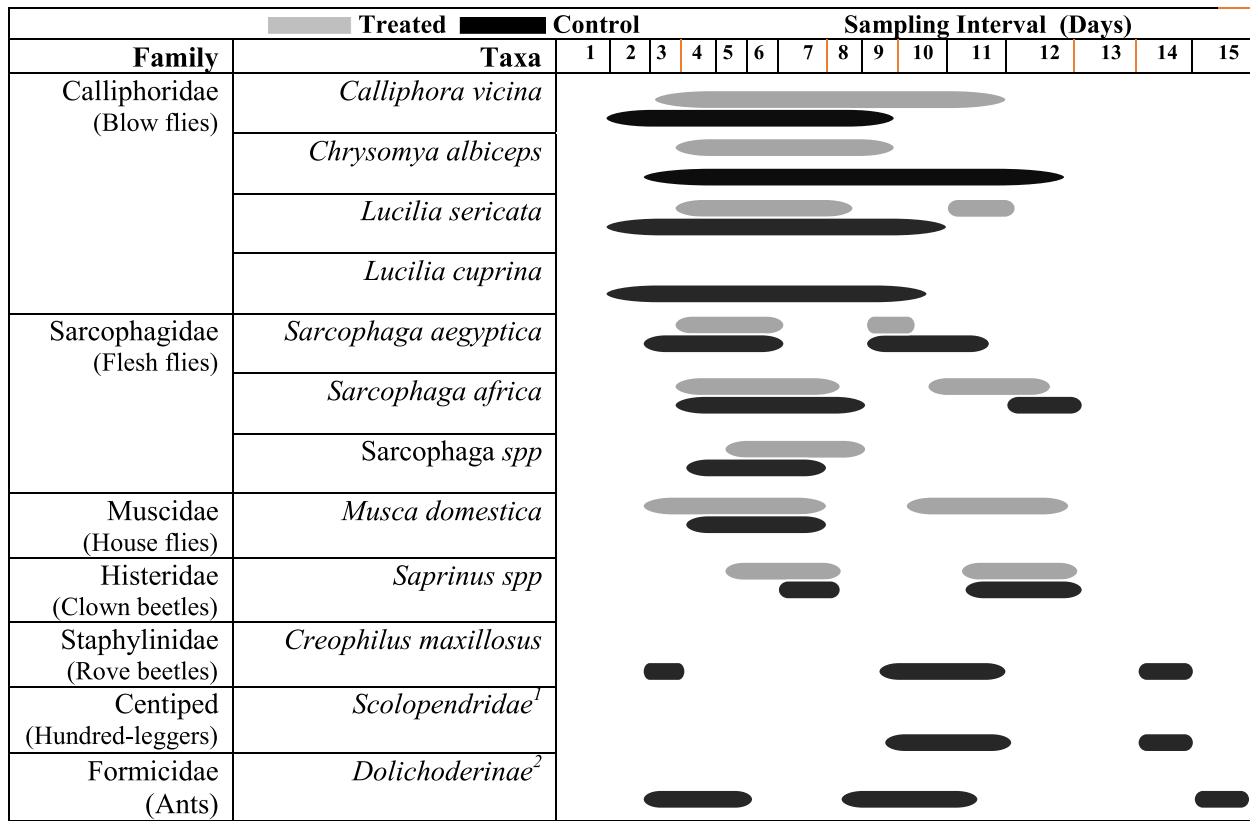
treated rabbits. *Creophilus maxillosus* was recorded only from the remains of untreated carcasses during the winter.

Ants (Formicidae) were mostly present on the control carcasses with large numbers at early stages of decomposition, and they were predated on eggs and take carcass pieces as food. *Messor spp.* (Formicidae) was observed on untreated carcasses until the end of the experiment in spring, the number of which had steadily decreased. The mean pairwise taxa similarities for insect taxa succession waves for each sampling day are shown in Fig. 3. The taxa resemblance values for 15 days sampling intervals during the spring trials ranged from 0.0 to 0.28 (0.16 ± 0.043) and 0.05 to 0.33 (0.21 ± 0.032) for untreated and treated carcasses, respectively. During the winter trials, these similarities ranged from 0.0 to 0.33 (0.22 ± 0.021) and 0.0 to 0.31 (0.18 ± 0.037) for untreated and treated carcasses, respectively. The lowest taxa resemblances were observed on the first day of

decomposition, but the greatest resemblances were recorded on days 3–5. Correlation analysis was used to test species resemblance values between sampling intervals. Permutation analysis based on the Mantel test were 0.64 ± 0.16 (P = 0.009) and 0.69 ± 0.16 (P = 0.003) for both treated and untreated groups in spring and winter, respectively. Therefore, the correlations were significant at the 0.05 level, and therefore, the null hypothesis was rejected. Permutation of similarity analysis was 0.82 ± 0.18 (P < 0.001) between the spring and winter. This finding showed that insect succession patterns were similar between the spring and winter.

Permutation analyses between the similarity values of the two most dominant fly species (*Ch. albiceps* and *C. vicina*) for both treated and untreated carcasses in the spring and winter were 0.51 ± 0.15 (P = 0.05) and 0.49 ± 0.14 (P = 0.09), respectively. Interestingly, the result showed that the succession patterns of the two species on the remains of both treated and untreated rabbits

**Table 2** Insect\* succession patterns for methadone-treated and untreated rabbit carcasses during 15 sampling intervals in winter 2019



1. A family of Centipedes or "hundred-leggers"

2. A subfamily of ants

\*Immature stages of flies, adult beetles, ants, and centipedes

were different. *Chrysomya albiceps* and *C. vicina* prefer to lay eggs on the control carcasses earlier than the treated carcasses.

**Discussion**

In the present study, the duration of decomposition process in the spring was faster. This could be due to higher temperatures in the spring. Temperature is an important factor in the carcass decomposition process and it gets faster as the temperature rises (Mashaly and Al-Mekhlafi, 2016). Various studies in different

geographical areas revealed that the decomposition processes were accelerated during the warmest months of the year (Koffi et al. 2017; Sanford, 2017; Keshavarzi et al. 2019).

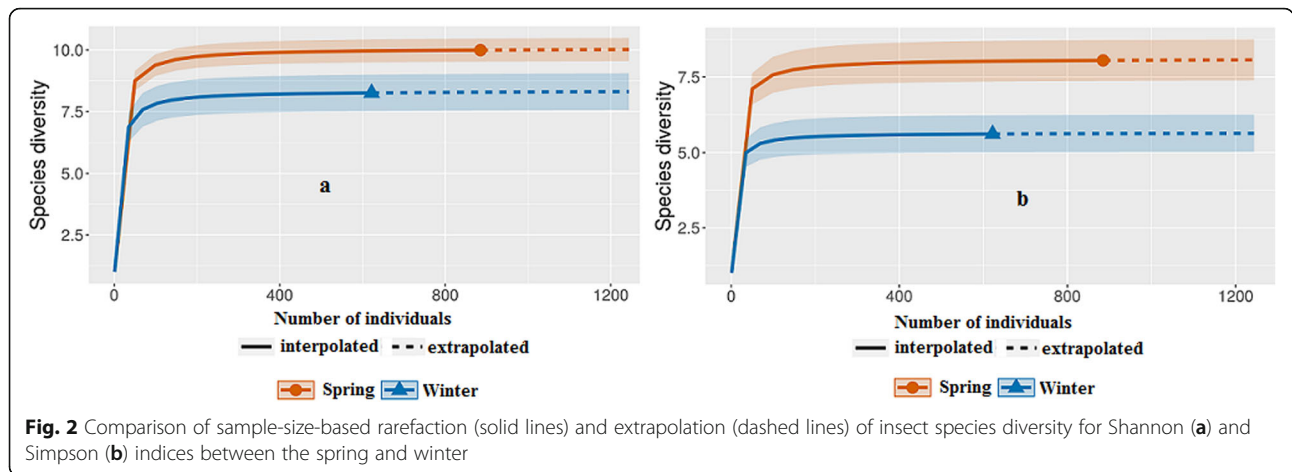
Our study showed that *C. vicina* activity was not inhibited on rainy days, while the activity of some species such as *L. sericata* was reduced. Similar result was reported by Keshavarzi and et al. (Keshavarzi et al. 2019). Reibe and Madea reported that ambient temperature and rainfall had no significant effect on the number of egg batches (Reibe and Madea, 2010).

Adult of calliphorids were the first visitors to both carcasses; this observation was in agreement with previous studies (Mashaly and Al-Mekhlafi, 2016; Keshavarzi et al. 2019). *Calliphora vicina* and *Ch. albiceps* were the dominant species and constituted the primary colonizer in the spring and winter, respectively. Abd El-bar and Sawaby reported that *Ch. albiceps* is a dominant species and also a primary visitor on carcasses of rabbits treated with an Organophosphate insecticide (El-Bar and Sawaby, 2011). We used immatures to study the trend of succession and according to Smith report, the presence of adult flies does not necessarily mean that egg laying

**Table 3** Mean duration (days) of the different decomposition stages in days for methadone-treated and untreated carcasses

The stages of decomposition	Mean duration (days) in spring		Mean duration (days) in winter	
	Treated	Untreated	Treated	Untreated
Fresh	1.7 ± 0.25	1.7 ± 0.25	2.7± 0.25	2.7± 0.25
Bloated	3.2 ± 0.25	3.2 ± 0.25	4.5 ± 0.50	4.5± 0.50
Decay	4.5 ± 0.00	4 ± 0.00	5.7± 0.25	5± 0.00
Post decay	6 ± 0.00	5.5 ± 0.50	7.5± 0.50	7± 0.00
Dry	9 ± 0.00	9 ± 0.00	12.5± 0.50	12.5± 0.50



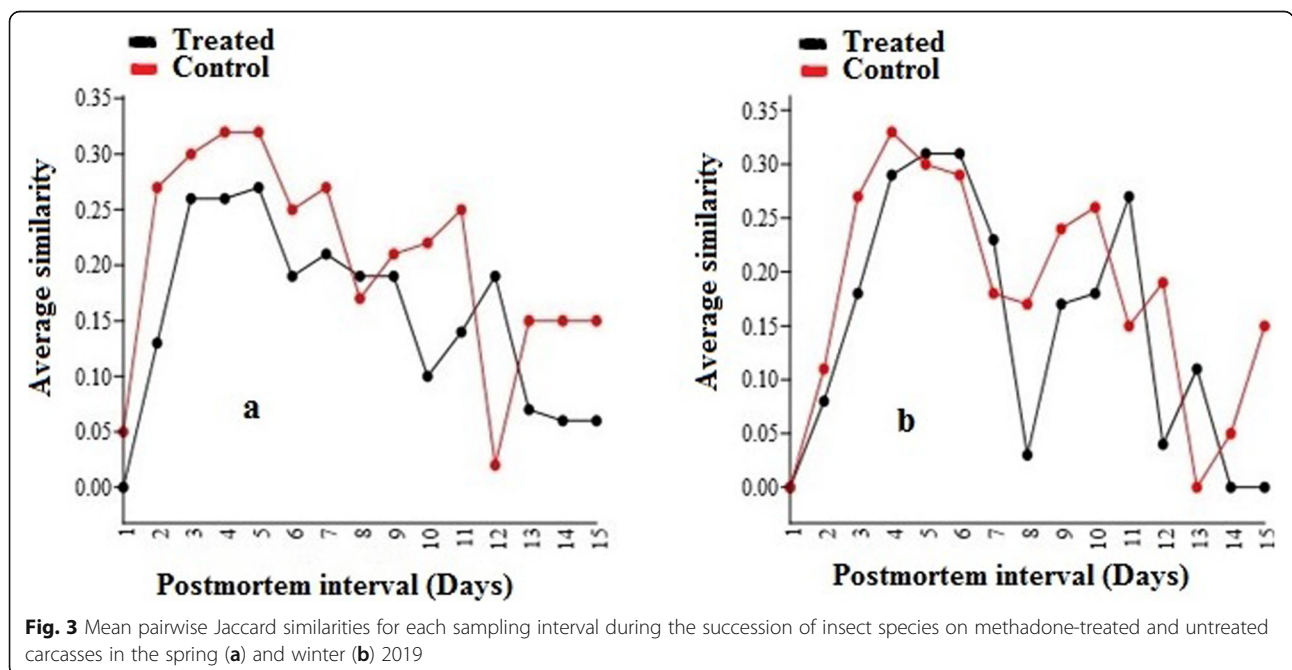


or larviposition occurs (London and Smit, 1957). Most larvae were found on untreated carcasses, but the most activity of adult flies was observed in the vicinity of the treated carcasses during the fresh and bloated stages. There was no significant difference between the mean number of larvae on untreated and treated groups ( $P = 0.28$ ). Dissimilar findings have been reported by Abd El-bar and Sawaby (El-Bar and Sawaby, 2011). Adult female flies choose safe sites for larvi-or oviposition due to the presence of xenobiotics, predators, and competitors (Archer and Elgar, 2003; Mahat et al. 2009). Mahat et al. (2009) observed that Malathion generally delayed the initial oviposition of dipteran species. Therefore, the presence of different substances in the remains can lead to different behavior in flies. Therefore, the greatest activity of adult flies in the vicinity of the treated carcasses

may be due to their efforts to choose the right breeding site for larvi- or oviposition.

*Lucillia sericata* was caught on day 2–6 as well as day 8–10 in the winter, but it was not caught on day 7. This event was also seen for the presence of other species during the spring and winter. This phenomenon has been reported in various studies [5, 16, 26]. This phenomenon is probably often due to sampling error, as various factors cause this error. For example, interspecies competition and ambient temperature may be leading to migration of insect species from the surface of the carcasses to the viscera, where sampling is not easy (Campobasso et al. 2001).

Some insects (beetles and ants) visit but do not colonize a carrion; rather, they use the carcass and dipteran larvae as food for both themselves and their



developing larvae. Some Coleoptera families such as Staphylinidae, Histeridae, and Dermestidae are valuable in succession-based PMI estimations (e Castro et al. 2013).

In this study, two beetle species (*Hister* sp and *Creophilus maxillosus*) appeared in the bloated stage in the seasons. But, the majority of beetles were observed during the decay and post-decay stages. *Dermestes maculatus* was active on the both carcass types during the final stages of decomposition. Keshavarzi and et al. analyzed arthropods succession on rat carcasses and showed that *D. maculatus* is active mainly during the post-decay and dry stages of decomposition (Keshavarzi et al. 2019). In this study, ants were more present in the early stages of decomposition, and mainly fed on carcasses. Similar finding has been reported on remains of rabbits treated with an organophosphate insecticide in El-Qalyubiya Governorate of Egypt (El-Bar and Sawaby, 2011).

Our study showed that the taxa resemblance values for 15 days sampling intervals ranged from 0.0–0.31 and 0.05–0.33 for untreated and treated carcasses, respectively. These results showed a low similarity in species between sampling intervals within each group, similar to Tabor et al. for ethanol-treated and untreated pigs (Tabor et al. 2005). The low degree of similarity reveals rapid temporal changes the composition of the taxa. Consequently, the slowest turnover in the composition of the taxa occurred on days 4–5. Based on our findings the overall pattern of insect succession was identical between the treated and untreated rabbit carcasses ( $P < 0.05$ ). At almost the same, Tabor et al. found that successional patterns of insect species were similar between ethanol-treated and untreated carcasses (Tabor et al. 2005). Similar findings have been reported in previous studies of carcasses treated with organophosphate (Yan-Wei et al. 2010; El-Bar and Sawaby, 2011). In the present study, statistical analysis showed that the succession patterns of *Ch. albiceps* and *C. vicina* were different between treated and untreated carcasses. This suggested that the antemortem use of methadone had an impact on the occurrence of these species in carcasses. According to Tabor et al. *C. vicina* tended to colonize the ethanol-treated carcasses as compared to control carcasses (Tabor et al. 2005). Differences in the successions of these two species between the carcasses could be due to the effect of methadone on the time of oviposition. The effect of methadone on the growth rate of flies can also be considered. Because, as mentioned before, this opioid reduces the developmental time of *C. vicina*. This means that the larvae started to migrate away from the treated remains earlier than the control carcasses. Therefore, this event can affect the succession pattern. Another hypothesis is that, methadone accumulated inside the tissues of the treated rabbit carcasses decreased the necrotic odor of the carcass's tissues and

subsequently leading to lower attraction of those flies. However, this hypothesis requires further investigation. It has been reported that adults and larvae of insects were more frequent on the warfarin-treated carcasses than in the control carcasses (El-Gawad et al. 2019). Abouzied (2016), observed that tramadol treated carcasses are more favorable for females of some fly species (AbouZied, 2016). Therefore, it seems that the presence of flies on carcass could be affected by the drugs in the carcass tissues.

Both of *Ch. albiceps* and *C. vicina* species, often found during cooler seasons and they were also the first wave invader during the decomposition of carcasses (Sanyanga, 2016; Feddern et al. 2018; Keshavarzi et al. 2019). Given that these species are present on both treated and untreated carcasses during the spring and winter, they can be used as a good indicator to estimate the time of death. But in the case of methadone abused cadaver, it should be noted that this substance have an effect on the PMI estimation based on the pattern of succession. Further study on the effect of methadone on the growth rate of both species is required. Methadone accumulates in different parts of the body of flies, such as adipocytes (in larvae), cuticle (in puparia), and meconium (in adults) which could act on insect physiology (Gosselin et al. 2011).

## Conclusion

Finally, we conclude that the overall pattern of insect succession was similar between the treated and untreated rabbit carcasses. But the patterns of the succession of two most dominant fly species (*Ch. albiceps* and *C. vicina*) were different between both treated and untreated carcasses. Those two species did prefer to lay eggs on the control carcasses earlier than the treated carcasses, and this could have an effect on the minPMI estimation based the pattern of succession. The differences observed in the patterns of the succession of *Ch. albiceps* and *C. vicina* on treated and untreated rabbit were sufficient to alter post-mortem interval estimates based on successional patterns by 24 h. For example, when larvae in the migratory phase or pupae are recovered from the scene, their age can be estimated using their development time. The estimated age indicates the minPMI, if we do not consider the effect of methadone on the initial oviposition of species on the corpse, the estimated time has a 24-h error. The current study provided a database in the field of medico-legal entomology that could be useful for estimating minimal post-mortem intervals.

## Abbreviations

C: Calliphora; Ch: Chrysomya; D: Dermestes; L: Lucillia; M: Musca; S: Sarcophaga; PMI: Post-mortem interval; Min: Minimum

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### Authors' contributions

KD, RS, PSZ, and RY planned the entomological sampling and performed the species identification. OMA and AA wrote the manuscript and calculated diversity in collaboration with AK. All authors read and approved the final manuscript.

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### Availability of data and materials

All data are available at School of Public Health, Tehran University of Medical Sciences.

### Declarations

#### Ethics approval and consent to participate

Necessary ethical approval was obtained from School of Public Health, Tehran University of Medical Sciences Ethics Committee.

#### Consent for publication

Not applicable

#### Competing interests

The authors declare that they have no competing interests.

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