Basic Medical Research

Insects on pig carcasses as a model for predictor of death interval in forensic medicine

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ABSTRAK

Latar belakang: *Entomologi forensik belum dimanfaatkan sebagaimana mestinya di Indonesia. Keberadaan serangga forensik di Indonesia juga belum banyak dilaporkan. Penelitian ini bertujuan untuk mendapatkan jenis-jenis serangga pada bangkai hewan coba yang dapat dipergunakan untuk perkiraan saat kematian.*

Metode: *Empat babi domestik yang dimatikan dengan cara berbeda digunakan sebagai model. Bangkai diamati dua kali sehari (sekitar pukul 09.00 dan 16.00) selama 15 hari untuk mendapatkan tahap-tahap dekomposisi dan koleksi serangga baik imatur maupun dewasa. Serangga imatur dipelihara dan serangga dewasa diidentifikasi di Laboratorium hama dan penyakit tanaman, Universitas Sam Ratulangi, Manado. Chrysomya megacephala dan C. rufifacies diidentifikasi baik secara morfologik maupun tehnik* deoxyribose-nucleic acid *(DNA).*

Hasil: *Lima tahap dekomposisi didapatkan dalam penelitian (segar, pembusukan awal, pembusukan aktif, pembusukan lanjut, dan skeletonisasi). Serangga yang ditemukan ialah 11 spesies Diptera and delapan Coleoptera selama 15 hari suksesi. Chrysomya megacephala, C. rufifacies dan Hermetia illucens berkolonisasi pada semua bangkai.*

Kesimpulan: *Pada keempat ekor babi serangga yang ditemukan terutama terdiri dari Diptera dan Coleoptera. Chrysomya megacephala, C. rufifacies dan Hermetia illucens tampaknya merupakan kandidat utama untuk perkiraan saat kematian.*

ABSTRACT

Background: Forensic entomology has not been acknowledged in Indonesia so far. Indonesian carrion insects are very rarely reported. The aim of this study was to obtain the types of insects on pig carcasses that could be used for the estimation of post-mortem interval.

Methods: Four domestic pigs sacrificed with different methods were used as a model. The carcasses were observed twice daily (around 9 a.m and 4 p.m) during 15 days to assess the stages of decomposition and to collect insects, both in mature and immature stages. The immature insects were reared and the mature insects were indentified in the Laboratory of Pests and Plant Diseases, University of Sam Ratulangi, Manado. *Chrysomya megacephala* and *C. rufifacies* were identified both morphologically and with deoxyribose-nucleic acid (DNA) techniques.

Results: Five stages of decomposition (fresh, bloated, active decay, post-decay, and skeletonization) were observed. A total of 11 Diptera and 8 Coleoptera species were found during a 15-days succession study. *Chrysomya megacephala*, *C. rufifacies* and *Hermetia illucens* colonized in all carcasses.

Conclusion: Insects found on four different pig carcasses consisted mainly of widespread *Diptera* and *Coleoptera*. *Chrysomya megacephala*, *C. rufifacies* and *Hermetia illucens* seemed to be primary candidates for the estimation of the post-mortem interval.

Keywords: *Chrysomya*, forensic entomology, *Hermetia*

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Insects have been used in forensic investigation as early as the thirteenth century in China.[1] The first serious description of insect succession on corpses was given by Mégnin.[2] Early forensic work on insects was done by Nuorteva[3] in Finland and Leclercq[4] in Belgium. After the publication of the first handbook by Smith[1] in 1986, forensic entomology became gradually more fashionable in normal police work. In the last three decades forensic entomology developed rapidly, especially in the areas with a temperate climate. More recently studies on this topic have been carried out in several tropical countries. As the carrion insects in most tropical countries are not very well documented, thus the findings of those insects will be a great challenge. So far, no specific forensic entomology studies have been conducted in Indonesia. Research was done on the island of Sulawesi, which is on the transition of the Oriental and Australian biogeographical regions. Sulawesi has a very peculiar fauna, rich in endemic species. During the Wallace project in 1985[5] carrion flies were collected by Kurahashi, et al[6-9] and carrion beetles by Hanski and Krikken[10], and Hanski and Niemela.[11] These insects were studied mainly in undisturbed rainforest habitats. Most human corpses are however found in an urban or suburban environment and the carrion fauna of this habitat type was not examined in Sulawesi on a systematic way.

According to the information from the Department of Forensic and Medicolegal Prof. Dr. R. D. Kandou General Hospital Manado, the most common cases of violent deaths in Manado are blunt stroke on the occipital area and cardiac puncture. In order to test whether the cause of death influenced the insect succession, four pigs were sacrificed in different ways. The aim of the present study was to identify the insect succession on pig carcasses as a model in forensic entomology for predictor of death interval.

METHODS

Field studies were carried out at Winangun and Batu Kota, Manado, North Sulawesi, Indonesia, between January 27th and February 15th, 2012. Winangun and Batu Kota are located at a latitude 1°26'41"N and longitude 124°50'11"E, respectively, at an altitude of 66 metres above sea level. Four domestic pigs weighing approximately 20 kg each (aged two month old) were used as a model. The 1st pig was sacrificed by using 200 g potassium cyanide per oral designated as carcass I; the 2nd pig was sacrificed by a blunt stroke on its occipital area, carcass II; the 3rd and 4th pig were sacrificed by cardiac punctures, carcass III and IV. After death, three pigs were placed in protective cages at a distance of 20 m from each other at Winangun, and the other one (carcass IV) at Batu Kota. The cages consisted of a wooden frame (1.6x1x1 m^3) were covered with wire meshing (diameter ± 1.7 cm).

The carcasses were observed twice daily (around 9 a.m and 4 p.m) during 15 days to assess the stages of decomposition (fresh, bloated, active decay, post-decay or skeletonization) and to collect insects.

Both mature and immature insects were collected. Insects flying around the carcasses or pearched on the carcasses were collected with an insect net and recorded. Immature insects including eggs, larvae in natural cavities and wounds, and larvae from other parts of the carcasses were collected for rearing and identification in the Laboratory of Pests and Plant Diseases, Sam Ratulangi University, Manado. Soil around and underneath the carcasses were examined to observe the presence of insects including pupae. Immature insects were reared on slices of fresh pig liver. Adult insects were sacrificed with ethyl acetate and mounted on entomological pins, while immature specimens were stored in 95% alcohol.

Specific literature for the identification of adult Sulawesi carrion insects is mainly limited to blowflies. Most other species were identified by direct comparison with identified museum material. The identification of immature insects is based on rearing to adults. Voucher specimens were deposited at the Sam Ratulangi University, Manado; and Naturalis Biodiversity Center, Leiden.

Chrysomya megacephala and *C. rufifacies* were identified both morphologically and with deoxyribose-nucleic acid (DNA) techniques. Samples for DNA identification were extracted with AxyPrep Multisource Genomic DNA Miniprep Kit. This kit uses column purification technique that extracts both core and mitochondrial DNA.

After extraction, samples with 1270 bp cytochrome oxidase I (COI) gene were amplified with polymerase chain reaction (PCR) using primer for *Chrysomya*, as follows:[12]

- C1-J-1718f (5'–GGAGGATTTGGAAATTGATTAGTTCC)
- TL2-N-3014r (5' –TCCAATGCACTAATCTGCCATATTA)
- TL2-N-3014MODr (5' –TCCATTGCACTAATCTTGCCATATTA)

Table 1. Steps of PCR of COI gene

Step	Temperature	Time
Predenaturation	95°C	2 minutes
Denaturation	95°C	30 seconds
Primary annealing	54°C	30 seconds
Extention	72°C	90 seconds
Final extention	72°C	90 seconds

Primers were made at PT Genetika Science Indonesia located in Jakarta, Indonesia. PCR was carried out with Biometra® T Personal, with temperatures of each cycle as seen as Table 1.

PCR products were visualized using 1.0% agarose gel, dyed in ethydium bromide 1.5% and transiluminated with ultraviolet (UV) rays, and were then sequenced with COI gene bar coding that is commonly used in flies identification.

RESULTS

The mean daily temperature at the study sites fluctuated between 23.4°C and 27.2°C. Almost all days had heavy rainfalls.

No comprehensive review of Indonesian carrion insects did exist. Many tropical carrion insects were described a long time ago without mentioning

Table 2. Sulawesi carrion insects reported in taxonomic literature

	Species	Source
Coleoptera		
Hybosoridae	*Phaeochrous emarginatus* Castelnau	Hanski & Krikken, 1991[10]
Hybosoridae	*Phaeochrous sulawesi* Kuijten	Kuijten, 1978[13]
Scarabaeidae	*Onthophagus aper* Sharp	Hanski & Krikken, 1991[10]
Scarabaeidae	*Onthophagus mentaveiensis* Boucomont	Hanski & Krikken, 1991[10]
Scarabaeidae	*Onthophagus scrutator* Harold	Hanski & Krikken, 1991[10]
Silphidae	*Necrophilia renatae* (Portevin)	Ruzicka, Schneider, Qubaiová & Nishikawa, 2012[14]
Silphidae	*Necrophilia charon* Sikes & Madge	Sikes, Madge & Trumbo, 2006[15]
Silphidae	*Nicrophorus distinctus* (Grouvelle)	Sikes, Madge & Newton, 2002[16]
Diptera		
Calliphoridae	*Calliphora hasanuddini* Kurahashi & Selomo	Kurahashi & Selomo, 1997[9]
Calliphoridae	*Chrysomya greenbergi* Wells & Kurahashi	Wells & Kurahashi, 1996[17]
Calliphoridae	*Chrysomya nigripes* Aubertin	Singh, Kurahashi & Wells, 2011[18]
Calliphoridae	*Chrysomya rufifacies* (Macquart)	James, 1977[19]
Calliphoridae	*Chrysomya yayukae* Kurahashi & Magpayo	Kurahashi & Magpayo, 1987[8]
Calliphoridae	*Hemipyrellia ligurriens* (Wiedemann)	James, 1977[19]
Calliphoridae	*Idiella divisa* (Walker)	James, 1977[19]
Calliphoridae	*Isomya delectans* (Walker)	James, 1977[19]
Calliphoridae	*Lucilia adisoemartoi*	Kurahashi, 1988[6]
Calliphoridae	*Lucilia papuensis* Macquart	James, 1977[19]
Calliphoridae	*Phumosia abdominalis* Robineau-Desvoidy	James, 1977[19]
Calliphoridae	*Phumosia elegans* Kurahashi	Kurahashi, 1989[7]
Calliphoridae	*Phumosia indica* (Surcouf)	James, 1977[19]
Calliphoridae	*Phumosia njonja* Kurahashi	Kurahashi 1989[7]
Calliphoridae	*Phumosia promittens* (Walker)	James, 1977[19]
Calliphoridae	*Strongyloneura prolata* (Walker)	James, 1977[19]

that they were collected on carrions. More recent taxonomical papers usually contain more ecological information but it is very difficult to trace specific carrion insects in scattered publications. Based on the carrion-taxa found in neighbouring countries, literature was searched for potential carrion insects in Sulawesi. The results of the literature search are summarized in Table 2.

Five stages of decomposition were distinguished in all carcasses. The duration of the different stages is illustrated in Table 3. The fresh stage was observed on day one. The bloated stage was observed on days two and three for all carcasses, but facial destruction was first observed on carcass II. The active decay stage was observed on days four and five in carcass II, III and IV meanwhile in carcass I on days four to six. The post-decay stage was observed on days six to eight in carcass II and III, and just on day six in carcass IV. In carcass I, however, this stage lasted until day 10. Skeletonization was observed on day nine for carcasses II and III, on day seven for carcass IV, but on day 11 for carcass I. The different insects in relation to the decomposition stages are summarized in Table 4.

Fresh stage

C. megacephala and *C. rufifacies* visited the carcasses at the fresh stage from day one. Eggs of *Chrysomya* were found on day one in natural orifices (mouth, nose, ear, and anus) and the stab wounds. Other *Diptera* which visited the carcasses on the same day were *Ophyra spinigera* and *Sarcophagidae*. *Formicidae* were found from the first hour of post-mortem in the natural orifices, especially in those with fresh blood.

Bloated stage

Blowfly larvae invaded the carcasses' faces voraciously especially in carcasses II, III and IV. *C. rufifacies* and *C. megacephala* were abundant. *Musca domestica*, *Ophyra spinigera*, and *Sarcophagidae* were still found at this stage. *Sarcophagidae* were observed only on intact skin, and not on the natural orifices or wounds. Beetles found at this stage were the predators *Saprinus* and *Philonthus* on day three. *Riptortus linearis* was found on intact abdominal skin on day three. On day two, Formicidae left the natural orifices invaded by *Chrysomya* maggots, but were still found in the non-invaded areas.

Active decay stage

At the end of this stage, *Diptera* larvae had consumed most part of the body tissues leaving the skin, cartilages, bones, and the last part of muscles and guts. This stage lasted for two days in carcasses II, III and IV, and three days in carcass I.

Table 3. Duration of decomposition stages in four different pig carcasses in Manado

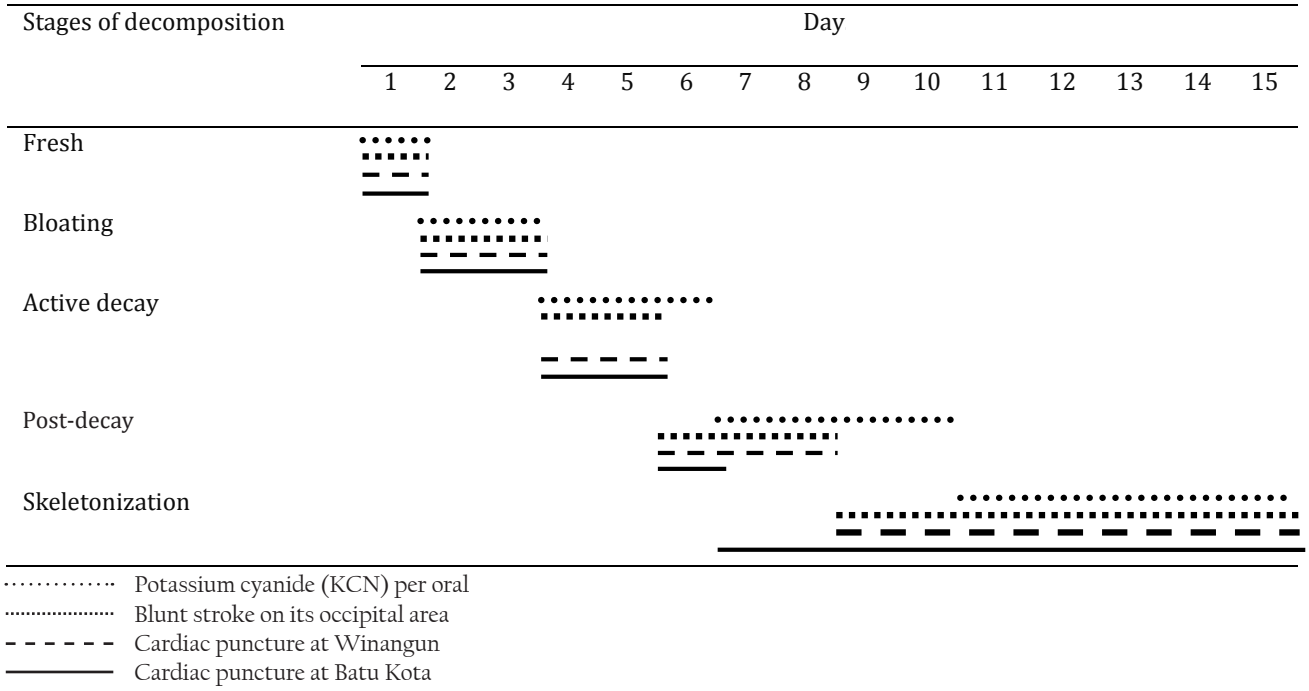

Table 4. Insects found on four different pig carcasses in Manado in relation to the decomposition stages

	Species	Fresh	Bloated	Active decay	Post-decay	Skeletonization
Coleoptera						
Cleridae	*Necrobia rufipes (De Geer)*	-	-	-	**A**	**A**
Dermestidae	*Dermestes maculatus* De Geer	-	-	-	**A**	**A**
Histeridae	*Pachylister tabellio* Marseul	-	-	-	**A**	-
Histeridae	*Saprinus* sp.	-	**A**	**A**	**A**	**A**
Hydrophilidae	*Sphaeridium discolor* Orchymont	-	-	**A**	-	-
Silphidae	*Diamesus osculans* Vigors	-	-	**A**	**A, L**	**A, L**
Staphylinidae	*Creophilus flavipennis* Hope	-	-	**A**	**A**	**A**
Staphylinidae	*Philonthus* sp.	-	**A**	**A**	**A, L**	**A, L**
Dermaptera						
Labiidae		-	-	-	**A**	-
Diptera						
Calliphoridae	*Chrysomya megacephala* (Fabricius)	**A**	**A, L**	**A, L**	**A, L**	**A**
Calliphoridae	*Chrysomya rufifacies* (Macquart)	**A**	**A, L**	**A, L**	**A, L**	**A**
Calliphoridae	*Hemipyrellia ligurriens* (Wiedemann)	-	**A**	-	-	-
Fanniidae	*Fannia* sp.	-	-	-	**A**	-
Muscidae	*Atherigona* sp.	-	-	**A**	**A**	-
Muscidae	*Musca domestica* L.	-	**A**	**A, L**	**L**	**L**
Muscidae	*Ophyra spinigera* Stein	**A**	**A**	**A**	**A, L**	**A**
Phoridae	*Phoridae* sp.	-	-		**A**	
Piophilidae	*Protopiophila* sp.	-	-	**A**	**A**	**A**
Sarcophagidae	*Sarcophaga* s.l. sp.	**A**	-	-	-	**A**
Stratiomyidae	*Hermetia illucens* (L.)	-	-	**A**	**A**	-
Heteroptera						
Alydidae	*Riptortus linearis* (Fabricius)	-	**A**	**A**	**A**	-
Hymenoptera						
Formicidae		**A**	**A**	**A**	**A**	**A**

Notes: A = adults; L = larvae

C. rufifacies and *C. megacephala* both larvae and adults were abundant. *Musca domestica*, *Ophyra spinigera*, and *Sarcophagidae* still visited the carcasses. Other *Diptera* such as *Protopiophila*, *Phoridae*, and *H. illucens* were found at the end of the active decay stage (day five). Beetles in this stage were *Saprinus*, *Philonthus* and *Sphaeridium discolor*. There were many Formicidae, predating on *Chrysomya* larvae which left the carcasses for pupation. *R. linearis* were still present.

Post-decay stage

During this stage, especially *Dermestes* consumed the remaining tissue. Only very few adult *C. rufifacies* and *C. megacephala* were observed. Other *Diptera* (*Protopiophila*, Phoridae and *H. illucens*) and *R. linearis* were still found in the early post-decay stage (day six). The beetles of the active decay stage were still present in this stage as well as a few Formicidae. On day eight, one adult *Labiidae* was found in carcass II.

Skeletonization

In this stage, adult *C. rufifacies* and *C. megacephala* of the new generation emerged from their pupae on days 12 and 13. However, their larvae and *Muscidae* larvae were still found under and around the carcasses. *Dermestes maculatus, Saprinus*, *Philontus,* and *Necrobia rufipes* were still present.

DISCUSSION

Decomposition

Generally, five stages of decomposition are recognized.[20-23] Payne[24] reported six stages (fresh, bloating, active decay, advanced decay, dry and

remains) in Clemson, South Carolina. Archer and Elgar[25] reported only two stages (initial decomposition and advanced decomposition) from Victoria, Australia while Wang, et al[26] in South China distinguished four stages.

In our study we observed five stages of decomposition in all carcasses; the duration of the decomposition stages in the different carcasses was however not the same. Carcass I took 10 days for skeletonization, carcasses II and III only took eight days, and carcass IV six days. Carcass I had no wounds or external blood in contrast to carcass II (fresh blood that came out of the mouth), III and IV (stab wound); oviposition by blowflies on carcass I was therefore much later. The duration of decomposition (7-10 days) resembled to those reported by Wang, et al[26] in South China and Heo, et al[27] at Tanjung Sepat, peninsular Malaysia meanwhile Apichat, et al[28] in Phitsanulok, Thailand reported one day each for fresh, bloated, and active decay stages, but three days for post-decay stage and a much longer skeletonization period (days 8-31).

Generally, *Calliphoridae* are the first insects to lay eggs on a corpse or carcass which makes them the most important tool of the forensic entomologist.[1,29] In our study, *Chrysomya megacephala* and *C. rufifacies* were abundantly found in both adult and immature forms. *C. rufifacies* maggots are predators on *C. megacephala* maggots and their eggs are therefore deposited later than those of *C. megacephala*. These dominant *Chrysomya* species are the same as those from similar experiments in other parts of tropical Asia as reported by Heo, et al[27] in Malaysia, Apichat, et al[28] in Thailand and Wang, et al[26] in South China.

Rearing of larvae and pupae in the laboratory resulted in adult *C. rufifacies* and *C. megacephala.* From both larva and pupa samples of carcasses II and III, the adults emerged one day earlier than those of carcass I. Adult *Chrysomya* at the study site emerged one day later than those in the laboratory. This faster development was caused by the higher temperature in the laboratory (28°-30°C).

Heo, et al[27] reported that the second generation blowflies appeared on day-12 and -13 around the vegetation on the study sites, which are similar to our results. Larvae of *C. megacephala* and *C. rufifacies* were still present underneath and around the carcasses until skeletonization. According to Gunn[30] adult flies also feed on the carcass during the post-decay stage or skeletonization, but they did not initiate oviposition.

Hermetia illucens was originally restricted to the New World but it has spread to other regions, both tropical and temperate.[29,31] This species is not often found in corpses, and is reported to colonize carrion in an advanced stage: 20-30 days post-mortem[21,30,32] In our study, adult *Hermetia illucens* were already found on day five. Diclaro and Kauffman[33] reported that *H. illucens* larvae have six instars which needed 14 days, and a pupation period of two weeks. The rearing of soil around the carcasses collected on day 11, resulted in a second generation of *Hermetia illucens* on days 35-39. The growth of the larvae was in accordance with the findings of Tomberlin, et al.[34]

Sarcophagidae larvae are reported from large carrion including forensic cases.[29,35] In our study, adult Sarcophagidae were found in small numbers from day 1 until the end of the study, but no maggots or pupae were observed. As these species apparently did not develop in the carcasses they cannot be used as forensic indicators. The adult *Sarcophagidae* were not identified to species level.

Larvae of *Ophyra* (instar two and three) are predators of larvae *Musca domestica* and other Muscidae larvae.[1,29,36] In our study, adult *Ophyra spinigera* was found from day 1-8, and their larvae on day 6-14 together with other *Muscidae* larvae.

Adults of *Musca domestica* usually visit a corpse after the arrival of blowflies and flesh flies in both the active and post-decay stages. House flies are rarely attracted to fresh corpses, unless there is excreta or exposed gut present.[1,29,30] In our study, *Musca domestica* was found from day 2-5, earlier than that observed by Byrd and Castner[29], Gunn[30], and Smith[1]. The first adult fly emerged during rearing in the laboratory on day seven which is similar to that of Gunn[30] who stated that the life cycle of *Musca domestica* takes six to eight days at a relatively high temperature.

In our study, adults of *Phoridae* were found on day-5. Rearing in the laboratory of soil collected at day eight resulted in pupae and adult *Phoridae*

which shows that development of the Phoridae occurred on the carcasses. Unfortunately the identification of *Phoridae* is notoriously difficult which prevents their usage as a forensic indicator at the moment.

In general, adults of *Protopiophila* visit carcasses in an active state of decay and an early dry stage. Larvae, as well as the adults, are necrophagous.[29] In our study, adult *Protopiophila* were found on day 4-6 but their larvae were not found on the carcasses or during rearing of soil samples. Adult *Protopiophila* were not identified to species level.

The beetles found in our experiment did feed on maggots or on parts of the carcass that were not consumed by maggots and therefore, they arrived usually later than the blowflies. The earliest Coleoptera visiting the carcasses were *Staphylinidae* (day-3), followed by *Saprinus* sp. (day-3, -5, -6, -10); these taxa are predators[29] and their presence was in accordance with the abundance of *Diptera* larvae on the carcasses. *Sphaeridium discolor* was found on day-4 and -5. Most *Sphaeridium* species are dung specialists and they were probably attracted by the gut contents of the carcasses. *Dermestes maculatus* (day-6, -10) and *Necrobia rufipes* (day-10) were observed later in the succession. *Dermestes* species are usually found on dry carcasses in the wild. Almost all species are scavengers and feed on various types of dried animal tissue.[29] *Necrobia* sp. have a special preference for fatty tissues: their larvae are sometimes predacious.[37] The *Coleoptera* larvae collected are belong to *Diamesus osculans* and *Staphylinidae*.

Formicidae are social insects which are distributed all over the world. In tropical areas they are very abundant and can be found in all stages of decomposition as predators or omnivores feeding on both body tissue and exudate fluids.[1,30] The ants were observed in the first hour after the start of the experiment and during the whole decomposition process in accordance with the statements of Aggarwal[38] and Heo, et al[39]. Ants were usually found in the natural orifices, they will leave these areas after being invaded by the *Chrysomya*.

Adults of *Riptortus linearis* were found on day 2-6. These bugs landed on the intact abdominal skin of carcasses, and not on the natural orifices or the open wounds, which suggest they are accidental visitors. All Alydidae, the family to which *Riptortus* belongs, are apparently plant-feeders[1,40] but Payne[41] reported three species of *Alydidae* feeding on a pig carcass in the USA. The relation between *Coreidae* and carcasses seems doubtful at the moment.

Labiidae are widely distributed, especially in hot climates.[42] Several species are predators on other insects by seizing them with their forceps or are omnivorous.[29,40,42] On day eight one adult *Labiidae* was found on carcass II. At this moment we consider this condition as an accidental visitor.

Literature study revealed a total of 11 *Diptera* and eight *Coleoptera* species which were belong to the potential carrion fauna of Sulawesi. Most of these taxa were different from those of the literature study, probably because the literature data were mainly based on the forest environments. The suburban Manado list contains mainly widespread taxa while the species from the literature list mainly endemic species in Sulawesi.

Five stages of decomposition (fresh, bloated, active decay, post-decay and skeletonization) could be distinguished during the 15 days study. *Chrysomya megacephala* and *C. rufifacies* dominated the other insects in all carcasses. *Formicidae* were the first visitors and found during the whole study, but abandoned areas which were invaded by *Chrysomya* larvae. Although the insect succession patterns were generally similar in all carcasses, the duration of decomposition and the moment on which the second generation of *Chrysomya* adults appeared were not the same. Traumatic carcasses showed a shorter decomposition period and an earlier emergence of *Chrysomya* adults. Further experiments are needed to prove that these succession patterns are reliable enough to be used in court.

The morphological identification of *C. megacephala* and *C. rufifacies* was confirmed with DNA-techniques. The carrion fauna of a limited part of Sulawesi is certainly not representative for all regions in Indonesia. Further research in other regions will reveal much more species of the carrion-community and thus, can lay a fundamental for the development of forensic entomology in Indonesia.

In conclusion, during our succession study on pig carcasses in suburban Manado, 11 Diptera and 8 Coleoptera taxa were collected. Six of the collected taxa were collected as larvae on the field location and two additional taxa were reared to adults from soil samples collected at the field location. The fact that these species developed on the carcasses classify them as potential forensic indicators. From *Chrysomya megacephala*, *C. rufifacies* and *Hermetia illucens,* the development from larvae to adults in relation to the ambient temperature is already known and they seem therefore to be primary candidates for the estimation of the post-mortem interval in suburban Sulawesi.

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Conflict of interest
The authors affirm no conflict of interest in this study.

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