Morphological and molecular techniques for the differentiation of myiasis-causing Sarcophagidae

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Morphological and molecular techniques for the differentiation of myiasis-causing Sarcophagidae

by

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A thesis submitted to the graduate faculty in partial fulfillment of the requirements for the degree of

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Chapter 1. Introduction

The USDA National Veterinary Services Laboratories (NVSL) is composed of many laboratories that perform health testing for the import and export of animals, and diagnosis and surveillance for diseases and their vectors of veterinary importance. One of the NVSL laboratories is the Parasitology laboratory, which identifies ecto-, hemo-, and fecal parasites in an effort to stop introduction, reintroduction or spread of foreign parasites. The continuing need for identification of ectoparasites arose mostly from two USDA pest eradication programs, the Cattle Fever Tick Eradication Program and the Screwworm Eradication Program (SEP). The latter, which will be discussed further in a later paragraph, charged the Parasitology laboratory with identification of any myiasis-causing fly submitted to the NVSL.

The term myasis was first proposed by Reverend F. W. Hope (1840) to describe animal disease caused by dipterous larvae. The spelling of this term was later corrected to myiasis, *myia* being the correct Greek root for fly (Borror 1960). These infestations are most often caused by flies in the families Calliphoridae, Sarcophagidae, or Oestridae (James 1947). For this document, myiasis is defined as the infestation of tissues in living mammals by dipterous larvae (maggots). Furthermore, the scope of most of this document is restricted to myiasis-causing flies in the family Sarcophagidae, with emphasis on this family as it is represented in the data available at the NVSL.
Myiasis can be broadly categorized into accidental, facultative, and obligate types (James 1947). Accidental myiasis is a gastric ailment and is caused by maggots that are consumed by the host, often subsequent to deposition of maggots on the host’s food; *Bercaea africa* (Wiedemann) is a common example of a sarcophagid fly in this category. Facultative myiasis is caused by maggots that normally feed on carrion and feces. Sometimes, such maggots are deposited on necrotic host tissue or on feces clinging to a host; in such cases, maggots may begin feeding on healthy tissue once the necrotic tissue or feces have been completely consumed. Most sarcophagid and calliphorid myiasis flies belong in this category. Accidental myiasis can cause gastric and intestinal distress (James 1947) and, in severe cases, destruction of anal papillae and intestinal walls (Herms & Gilbert 1932) in humans and other animals. The validity of many putative intestinal myiasis cases is uncertain, but undoubtedly, some such observations are based on feces contaminated by maggots or fly eggs after defecation (James 1947). Obligate myiasis is caused by maggots that require feeding on living host tissues to develop; in Sarcophagidae, this group is represented, in part, by some flies in the genus *Wohlfahrtia*, and obligate myiasis flies also occur in the Calliphoridae and Oestridae.

Facultative myiasis is the most commonly encountered form of maggot infestation submitted to the NVSL. This form of myiasis, sometimes called wound or traumatic myiasis, is often the result of reproductive female flies attracted to the smell of fresh or infected wounds. If it is not treated promptly, facultative myiasis can result in
damage to fur, leather, and meat products, and with enough time, death of the host is possible (Baumgartner 1988).

Obligate myiasis often results in the loss or damage of many wild and domesticated animals. All flies in the family Oestridae are exclusively parasitic on mammals as larvae (Wood 1987). Species of oestrid flies show a high level of host specificity, and in many cases, individuals of a species infest the same anatomical area on each infested host (Sabrosky 1986) (e.g., maggots in the subfamily Gastrophilinae are obligate parasites in the stomachs of mammals). Oestrid infestations can lead to damaged leather and fur in livestock animals. Additionally, heavy infestations of nasal bot flies and stomach bot flies can be severe enough to cause death in wild or domestic hosts (Zumpt 1965, Wood 1987).

The family Calliphoridae has two obligate myiasis species, the screwworms, *Chrysomya bezziana* Villeneuve in the Old World and *Cochliomyia hominivorax* (Coquerel) in the New World. New World screwworms historically were the chief myiasis-causing flies in North America. By early 1957, livestock producers in the southeastern U.S. were losing over $153 million (2010 dollars) annually to these flies (Meadows 1985). More recently, it was estimated that reintroduction of the screwworm to the U.S. would result in losses of more than $900 million annually (Collazo-Mattei 2011). Losses like these prompted the U.S. Department of Agriculture to begin the innovative SEP, with the first releases of sterile male flies occurring in Florida later in 1957. As a result of this successful effort and its
successors, wild screwworm populations presently exist in only South America and some Caribbean islands.

Four species of sarcophagid flies in the genera *Wohlfahrtia* and *Neobellieria* are obligate parasites of mammals. The Old World *Wohlfahrtia* species maggots enter cuts, scratches, or other breaks in the host skin. Nearctic *Wohlfahrtia vigil* (Walker) can enter a host through unbroken skin, but only thinner skin, such as that of young animals is vulnerable (James 1947). Infestation of young fur-bearing animals by *Wohlfahrtia* spp. can result in damaged pelts or even death. American fur farmers raising mink and fisher have experienced 20 – 30% animal infestation rates, with annual losses of as much as $4,000 per farmer in 1941 (Knowlton 1941, Strickland 1949). *Neobellieria citellivora* (Shewell) has been verifiably documented to infest only western North American ground squirrels, *Urocitellus columbianus* (Ord) and *U. richardsonii* (Sabine), with deaths resulting in some cases (Shewell 1950, Michener 1993). Both of these squirrels serve as food for various predators including ursids, canids, felids, mustelids, and some species of avian raptors, but they also feed on agricultural crops and compete with livestock for forage (Michener & Koepppl 1985, Elliott & Flinders 1991).

In addition to the available tools of eradication (e.g., mass-release of sterile males, quarantines, attractants, and insecticides) a surveillance program remains in place in the U.S. to detect screwworm incursions. Veterinarians and other cooperators encountering myiasis cases in the U.S. send maggot samples to the USDA NVSL Ames laboratories in order to identify or rule out potential screwworms.
The largest void in the NVSL entomologists’ ability to identify dipterous parasites to species is the maggots of the family Sarcophagidae. Between the years 1996 and 2006, inclusively, 48,195 cases of all kinds were submitted to the NVSL Parasitology laboratory for identification. Of these cases, nearly all of the non-myiasis cases were identified to the species level. Of the 1,349 (2.8% of the total cases) myiasis cases, 122 (9%) involved sarcophagid maggots. Discounting the sarcophagid cases caused by *Wohlfahrtia vigil*, which can be identified based on existing comprehensive description of its larval stages, only two of the remaining sarcophagid cases were putatively identified to species. The first case consisted of two, third instars found in the ear of a dog from Texas. The larvae were identified as *Titanogrypa pedunculata* (Hall), though the case report states that the identification was only to genus, with the tentative species name being based on the fact that it is the only species in its genus that has been implicated in myiasis. The second case involved a female *Liopygia crassipalpis* (Macquart) and 26 first-instar larvae. The inclusion of an adult with this sample made identification possible, but poor condition of the adult specimen made this identification tentative, as well. With proper descriptions and an adequate dichotomous key for sarcophagids, precise species level identifications of myiasis-causing maggots could be increased significantly (up to 9% based on previous submissions). Currently, such adequate descriptions for sarcophagid larvae do not exist. The objective of this thesis is to generate detailed descriptions of the species of myiasis-causing third-instar maggots of the family Sarcophagidae from North America north of Mexico.
The sarcophagid taxonomy in this document follows that used by Thomas Pape (1996), with the exceptions of Pape’s genera *Sarcophaga* and *Blaesoxipha*. Pape states in his catalog that phylogenetic resolution for this family is poor beyond the subfamilial level. This is especially true of the genera *Sarcophaga* and *Blaesoxipha*.

Of the 133 proposed *Sarcophaga* subgenera, Pape proclaims that 66 are monotypic. As such, radical changes in the systematics of the genus should be expected, and because of the large number of similar species in the genus *Blaesoxipha*, it has been proposed that the genus and its members might at some time be placed in their own family (Villeneuve 1908, Pape 1994). The natural implication of these observations is that the family Sarcophagidae may be more accurately divided into a greater number of genera. For practical convenience in this document, the *Sarcophaga* and *Blaesoxipha* subgenera of Pape’s catalog are presented at the level of genus.
In this section, each of the 19 species of sarcophagid flies previously implicated (Appendix 1) in North American myiasis cases are listed alphabetically and a general discussion of the species is provided. See Table 1 for a list of what are arguably the most notable sarcophagids in North America north of Mexico myiasis, based on an extensive review of the literature.

*Bercaea africana* (Wiedemann)

Even determining the correct name to use for this fly proved to be difficult because of a series of historical events that resulted in the widespread and continued use of the name “*Sarcophaga haemorrhoidalis*” for two different sarcophagid species. One of the possible synonyms for this fly taxon, *S. haemorrhoidalis* (Meigen), was not listed as a *B. africana* synonym in Pape’s catalog, but it was listed as a synonym of *S. georgina* Wiedemann in the catalog of North American Diptera (Stone et al. 1965); however, Pape did accept *S. georgina* as a synonym of *B. africana*. *Sarcophaga haemorrhoidalis* proves to be as enigmatic as it is important. The two separate entities that have carried the name *S. haemorrhoidalis* were a species described by Fallén and another described by Meigen. To compound the difficulties, *S. haemorrhoidalis* (Fallén) is listed in Pape’s catalog as a junior synonym of another fly name, *Ravinia pernix* (Harris).
According to Pape (personal communication), this confusion is the result of personal interactions between Fallén and Meigen. During a visit, Meigen examined Fallén’s collection, including Fallén’s own specimens of what was then described as *Musca haemorrhoidalis* (Fallén). After returning home, Meigen looked through his own collection and, from memory, misidentified a specimen as *M. haemorrhoidalis*, which he improperly credited to Fallén. Moreover, as the foremost authority on European Diptera, Meigen subsequently led many other authors to the same misuse of the name *Sarcophaga haemorrhoidalis* (in the sense of Meigen), to the point that it later was written as *Sarcophaga haemorrhoidalis* (Meigen) in many publications. In 1826, Meigen published a taxonomic revision of European Diptera, in which he proposed the new generic name *Sarcophaga* and placed *M. haemorrhoidalis* in this new genus. Pape (1986) later placed *S. haemorrhoidalis* (Fallén) as a junior synonym under the name *R. pernix* and *S. haemorrhoidalis* (Meigen) under *Bercaea cruentata*. Even more recently, Pape (1996) found an older name, *B. africa* (Wiedemann), to supersede *B. cruentata*. Because of the continued erroneous use of the name *S. haemorrhoidalis* in published literature, where either *R. pernix* or *B. africa* should be used, and the fact that *B. africa* is a cosmopolitan species, it can be difficult for readers to determine if such a literature reference pertains to *B. africa* or *R. pernix*.

This issue is made even more complicated by the widespread, almost indiscriminate misuse, of the name *S. haemorrhoidalis* as a catch-all for many other species of sarcophagid flies found in cases of myiasis (Zumpt 1965) and, similarly, for almost
any *Sarcophaga* maggots found on decaying carcasses. Such a lack of scientific
rigor may have led to additional frequent mistaken uses of the name *S.*
*haemorrhoidalis* in myiasis and forensic cases, in some instances, when neither *B.*
africa nor *R. pernix* is present.

Bryan (1937) claimed that, in a case of intestinal myiasis, *S. haemorrhoidalis* was
reproducing by paedogenesis. Paedogenesis is reproduction by an immature stage,
e.g., larval stage, of an organism. Bryan used the idea of paedogenesis to explain
continued intestinal infestation of a patient under conditions that he believed did not
allow reinfestation, i.e., the patient’s house and habits were free of flies. Herms and
Gilbert (1933) made a similar claim about another case of intestinal myiasis,
although this case was attributed to maggots from three different genera
(*Sarcophaga, Lucilia, Calliphora*). The contemporary presence of three species in
this case suggests multiple sources of infestation, which would invalidate Herms and
Gilbert’s claim that reinfestation was not a possibility. For validation, both of these
claims reference the experiments of Parker (1922), who claimed to show
paedogenesis in *Calliphora erythrocephala* (Meigen). This claim, however, was later
refuted by the more extensive experiments of Keilin (1924), who evidently was
unknown or ignored by Bryan (1937) and Herms and Gilbert (1933).

Maggots of *B. africa* are most often found feeding on feces, but they also have been
found on decaying meat and animal products (Aldrich 1916, Zumpt 1965). The
attraction of *B. africa* to almost anything moist and foul smelling, combined with its
nearly cosmopolitan distribution, lead me to hypothesize that this fly may be more

For *B. africa* entries in the detailed list of myiasis-implicated sarcophagid species in Appendix 1, references to *S. haemorrhoidalis* from only the New World were used; *R. pernix* is found in only the Old World.

**Gigantotheca plinthopyga** Wiedmann

Aldrich (1916) says many *G. plinthopyga* (=*Sarcophaga robusta* Aldrich) flies have been bred from carcasses and exposed beef, but at least nine published references implicated *G. plinthopyga* in myiasis cases, four of which involved myiasis in wild animals in Texas. Roberts (1931, 1933) gives case reports of two maggot-infested gunshot wounds in separate rabbits; one is identified as a Texas jack rabbit (*Lepus californicus texianus* Waterhouse), and the other is described only as a rabbit. No collection location is given for either of these rabbits, but the environs were described as dense mesquite brush. Lindquist (1937) describes other instances of *G. plinthopyga* infesting Texas jack rabbits. He gives almost no details of the cases, but does state that one rabbit was infested with only *G. plinthopyga*, and that *G. plinthopyga* was present, as a secondary infestation, in some of the other cases identified. Other than citing Roberts (1933), James (1947) gives no more details, and makes only the statement that *G. plinthopyga* has been reported infesting rabbits
and “other animals”. During a three year field study, Denno and Cothran (1976) found *G. plinthopyga* uncommonly infesting rabbit carrion traps in central California.

It should be noted that Lehrer (2010) recently proposed the resurrection of the sarcophagid genus *Hystricocnema* Townsend and the placement of *G. plinthopyga* in it. It is too soon for me to accept this move.

*Helicophagella melanura* (Meigen)

*Helicophagella melanura* are generalist feeders, with adults found on or near feces and carrion (Sanjean 1957), and larvae found feeding in decaying organic matter or as facultative parasites of mollusks, insects, birds, and mammals (van Emden 1954).

Specifically, case reports include of *H. melanura* infesting: the frenulum of the glans penis on a 43-year-old Japanese suffering from dementia (Miyamoto et al. 1996), the leg of a 66-year-old Japanese man suffering from tetraplegia and necrosis of the leg (Chigusa et al. 1997), and a Danish hedgehog (*Erinaceus europaeus* L.) piglet (Nielsen et al. 1978). Although this species occurs in the U. S. it has not been reported in myiasis from the U. S.

*Liopygia argyrostroma* (Robineau-Desvoidy)

This species ordinarily feeds on carrion in its immature stages, but it also is known to infest wounds of humans and other animals (James 1947). According to James (1947), *L. argyrostroma* can be either a primary or a secondary invader of tissues. The United States distribution (California, Indiana, Missouri, New York, North Carolina, Pennsylvania, and Texas) listed by Pape (1996) is largely disjunct and,
therefore, many of the connecting states are probably part of its distribution, including Iowa, from which this species has been collected and identified. Numerous publications conclude that this species is important in forensic entomology (Grassberger & Reiter 2002, Draber-Mońko et al. 2009, Niederegger et al. 2010).

**Liopygia crassipalpis** (Macquart)

This species of maggot is implicated mostly in wound myiasis (James 1947, Ali-Khan & Ali-Khan 1974, Uni et al. 2006) and intestinal myiasis (Riley 1939, Shiota et al. 1990). Exceptions include, first, Morris (1987) reports a case of aural myiasis in a mentally handicapped and nonresponsive teenage male human. In this case, there is not enough information to confirm or reject the identification. Second, is a Japanese case of ophthalmomyiasis in a bed-ridden elderly woman (Uni et al. 1999). A published photographic image depicting one of the reared adult flies, however, does not look like known *L. crassipalpis* adults from my own collection. This species of fly is used commonly in research pursuits, and a Zoological Record search for “Sarcophaga crassipalpis” yielded 98 citations from 1978 to the present, almost all for experimental reports.

**Liopygia ruficornis** (Fabricius)

Of the four primary references implicating this species in myiasis, none of the cases occurred in the United States. Two reports were from India (Bishopp 1915, Sinton 1921), one from “the Oriental region” (Patton 1923), and one from Brazil (Ferraz et al. 2010). The distribution of this fly (Pape 1996) suggests it is an Old World fly that
has invaded to the New World via human commerce and travel. Its New World
distribution is confined to islands and coastal countries, including Brazil, Panama,
and American Samoa. In the U. S., *L. ruficornis* is found only in states with maritime
ports (California, Florida, Massachusetts, New York, North Carolina, and
Pennsylvania) and Washington, D.C.

**Liosarcophaga sarracenioides (Aldrich)**

Bishopp et al. (1917) list this species in a footnote, along with *Rafaelia texana*
(Aldrich), and *G. plinthopyga*, as myiasis-causing flies. Aldrich (1916) cites rearing of
this species from orthopteroid, coleopteran, and lepidopteran hosts, as well as from
carrion but with no reference to myiasis. No other references to maggot feeding
behavior were found; therefore this species and its status as a myiasis fly remains
questionable.

**Liosarcophaga shermani (Parker)**

A single reference was found implicating *L. shermani* in myiasis of humans
(Baumgartner 1988). This reference, however, cites only James (1947) as evidence.
In fact, James implicates *Sarcophaga exuberans* Pandellé in myiasis of the eye but
does not give a host, and Baumgartner must have misconstrued the record as
representing *L. shermani*. Stone et al. (1965) state that references to *S. exuberans*
Pandellé in North America are not valid nomenclature, and thus, implied references
to *L. shermani* in myiasis probably refer to another fly species.
**Neobellieria bullata** (Parker)

Only routine, credible implications of this species in myiasis were found (Appendix 1). It is another species commonly used in research, and a Zoological Record search for “Sarcophaga bullata” yielded 292 citations from 1978 to the present, almost all for experimental reports. Also, specimens received from two commercial sources and one university laboratory for this study were identifiable as *N. bullata*.

**Neobellieria citellivora** (Shewell)

With the exception of one reported case of aural myiasis in a human (Curtis 1956), this species has been implicated in myiasis of two species of ground squirrels only. Although the evidence necessary to discount the human case, Curtis correctly states that adult *N. citellivora* and *N. bullata* are similar in appearance and even goes as far as suggesting that some records of *N. bullata* actually may be for incorrectly identified *N. citellivora*. It is possible that the fly identification used by Curtis was incorrect and his reported case was, in fact, caused by *N. bullata*. Furthermore, the data from Shewell (1950) and Michener (1993) suggest that *N. citellivora* is an obligate parasite, and that it feeds almost exclusively on *Urocitellus* ground squirrels.

**Neobellieria cooleyi** (Parker)

The only reference implicating this species in myiasis was in James (1947). He simply states that adult *N. cooleyi* were reared from maggots removed from a man in Saskatchewan. According to other references on this fly (Parker 1914, Denno & Cothran 1975, 1976), *N. cooleyi* is a scavenger of dead animal tissue; as such, it is
possible that the putative human myiasis case was accidental and anomalous or based upon a misidentification.

*Rafaelia texana* (Aldrich)

As with *L. sarracenoides*, the only reference that implicates *R. texana* in myiasis was a footnote in a published report (Bishopp et al. 1917). Aldrich (1916) includes a quote regarding *R. texana* from Bishopp, wherein he says this species was bred from carcasses and exposed beef. No other available reference to this species justifies its consideration as a myiasis-causing fly.

*Ravinia anxia* (Walker)

Dove (1937) implicates this species in three cases of intestinal myiasis. James (1947) says this species breeds in feces and that the cases reported by Dove (1937) were probably the result of stool samples contaminated after excretion.

*Sarcodexia lambens* (Wiedemann)

This species has been implicated in human and animal myiasis mostly in South America (Neiva & de Faria 1913, Bishopp 1915, Patton 1921, James 1947, Fernandes et al. 2009), although James (1947) claims without supporting evidence that *S. lambens* has been found infesting Florida cattle.

*Sarcophaga marionella* Aldrich

Knipling and Travis (1937) have this species in a list of flies they found to cause secondary myiasis in Georgia. A search of Pape (1996), Stone et al. (1965), Aldrich
(1916), Zoological Record, and other on-line sources, found no other mention of this species name. Because of this, it is likely that S. marionella Aldrich is a nomen dubium that cannot be definitively associated with any myiasis-causing fly.

**Titanogrypa alata (Aldrich)**

This species is cited by Townsend (1935, 1937, and 1942), James (1947), and Baumgartner (1988) as a myiasis-causing fly. Baumgartner cites the James reference; James in turn references Townsend’s papers. Townsend (1942) references part 2 of his Manual of Myiology (Townsend 1935), and Townsend (1935 and 1937) states that maggots in the genus Titanogrypa sometimes infest wounds, with few further details given. It is likely that James made the assumption that Townsend was referring to T. alata, which is the type species for this genus. Townsend (1942) does, however, cite Riley (1906), who makes the statement that Sarcophaga carnaria (L.) has been found infesting wounds and nasal and aural orifices. Townsend (1942) declares S. carnaria to be a synonym of T. alata, but he does not give justification for this action.

**Titanogrypa pedunculata (Hall)**

According to Stone et al. (1965) flies from Texas identified as Titanogrypa placida (Aldrich) are in fact T. pedunculata. According to Pape (1996), the distribution of T. pedunculata is Texas and Baja California Norte, Mexico, while the distribution of T. placida is from Panama north to El Salvador and in Sonora, Mexico. The fact that Sonora, Mexico, is disjunct from the rest of T. placida’s distribution, combined with
Sonora’s interstitial proximity to both Baja California Norte and Texas, may indicate that Sonoran specimens of *T. placida* are in fact *T. pedunculata*.

Based largely on the synonymy and geographic occurrence listed by Stone et al. (1965) and the habitus drawing of *T. placida* in Brill (1987), maggots in a single myiasis case submitted in 2000 to the National Veterinary Services Laboratories (accession #57466, case #SW00-184) were misidentified by J. W. Mertins as *T. pedunculata*. After further investigation in Greene (1925), the original source of the habitus drawing in Brill (1987), this identification was declared erroneous. As a result, there are no valid case reports implicating *T. pedunculata* in North American myiasis.

*Tripanurga sulculata* (Aldrich)

Roberts (1931, 1933) reported this species infesting wounds of jack rabbits (*Lepus californicus texianus*) in Texas, without indication of how the maggots were identified.

*Wohlfahrtia vigil* (Walker)

*Wohlfahrtia meigenii* (Schiner) and *W. opaca* (Coquillett) are the two most common synonyms of *W. vigil* in the literature, with the latter more prevalent. *Wohlfahrtia vigil* is one of the two obligate parasites on my list; *Neobellieria citellivora* is the other.

This species feeds almost exclusively on newborn and very young animals, through whose thin skin it is able to burrow to feed on the sub-dermal tissues. Historically, it was a common pest of many animals raised commercially for the fur trade (Winkler
1940, Strickland 1949, Gorham and Griffiths 1952, and others [see Appendix 1]).
According to Knowlton (1941), *W. vigil* caused annual losses per farm of up to $4,000 (about $60,000 today). Records have not been published after 1968 implicating *W. vigil* in myiasis of farm-raised fur animals in the U. S., perhaps because new cage designs and finer screening on the cages came into use, reducing the accessibility of *Wohlfahrtia* to larviposit on the newborns, or better pesticides came into use.

Many cases of human myiasis caused by *W. vigil* have been reported, but the first verifiable case was from an infant human in 1919 (Walker 1920). An earlier case (Washburn 1903) was identified as caused by maggots of *Gastrophilus epilepsalis* (French), which was later known as *Sarcophaga epilepsalis* (Pape 1996). The circumstances and other information given in the original Washburn report suggest that this case was, in fact, caused by *W. vigil*, and the putative *S. epilepsalis* maggots were misidentified. The most recent human case in the literature was in 1979 (Smith et al. 1981). Although a large number of reported *W. vigil* cases have human hosts (see Appendix 1), it could be that it is the personal nature of human cases that causes them to be written about more frequently. Perhaps the number of human cases has waned in recent history due to the increased use of screens on modern buildings and an increasingly urban-dwelling American population.

With simple precautions, such as the use of finer screening and insecticides reducing the numbers of myiasis cases in humans and farm-raised fur-bearing animals, *W. vigil* is reduced to using wildlife as primary hosts. Reports exist of *W.
vigil myiasis from 1921 (Shannon 1923) to 1998 (Schorr and Davies 2002) on various wild animals, including rabbits, coyote, and various rodents (see Appendix 1). More recently, cases of W. vigil myiasis have been submitted to the NVSL. These cases have been mostly from young domestic kittens and puppies, but one case was from young black-footed ferret (Mustella nigripes [Audubon and Bachman]) kits located in a wildlife preserve in New Mexico. The Turner Endangered Species Fund, (TESF) 2000 – 2001 biennial report, hints at problems W. vigil is causing by killing off newborn kits in its program of captive-rearing endangered black-footed ferret kits for release (TESF 2001).

All of these things considered, there are seven species of flies that should be represented in a key to myiasis-causing sarcophagids in North America north of Mexico (Table 1).

<table>
<thead>
<tr>
<th>Table 1. Species of Diptera in the family Sarcophagidae posing a significant threat of myiasis in the United States.</th>
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<tr>
<td>Bercaea africa</td>
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<td>Gigantotheca plinthopyga</td>
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<td>Liopygia argyrostroma</td>
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<td>Liopygia crassipalpis</td>
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<tr>
<td>Neobellieria bullata</td>
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<td>Neobellieria citellivora</td>
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<tr>
<td>Wohlfahrtia vigil</td>
</tr>
</tbody>
</table>

Methods and Procedures

Third-instar maggots were used in this study because they are the most common life stage submitted to the NVSL for identification. This is probably because a greater
amount of time is spent in this stadium than in other larval stages. It was, however, also important that the maggots used came from known, identified adult flies, because most of these species can be reliably identified only as adults.

Sources of the flies and maggots used in this study are listed in Table 2. The trap used to collect the two species in Ames, IA, was a plastic 2-liter soda pop bottle, with an approximately 5-cm-high by 15-cm-wide hole cut in the side to allow fly entry. It was baited with a mixture of raw meats, then left outside in a residential area, and checked daily for maggot deposition. All samples of *N. citellivora* were collected by a colleague from Richardson’s ground squirrels, *U. richardsonii*, in Alberta, Canada. The *G. plinthopyga* subjects were collected by a colleague from animal carcasses and raw beef liver in southeastern Arizona.

Flies were reared in the laboratory in a rectangular 40-liter glass aquarium covered with an acrylic lid that had a sleeved hole cut into it for access. A simple diet of sliced raw beef liver and sugar water was used to feed the adults. The liver was placed in a plastic Petri dish in the flies’ cage, and it also was used by female flies for larviposition. The liver was replaced each morning and checked for larvae. If maggots were present, the Petri dish with the liver was removed to a covered one-liter disposable plastic dish filled approximately one-third full with aspen-wood animal bedding. Several small pin-holes were punched in the one-liter container lid to provide air exchange and inhibit mold growth due to high humidity from the liver. Once the maggots matured and burrowed into the bedding to pupate, residual liver was removed from the dish, and flies were allowed to develop and emerge. With the
exception of *W. vigii* and *N. citellivora*, of which live specimens were not obtained, all maggots used for morphological and molecular study were the offspring of flies that were identified to species either before or after larviposition, i.e., F1 generation larvae. Adult flies were identified using morphological identification keys and descriptions found in several sources including: Aldrich (1916), Shewell (1950, 1987), and Pape (1994). Study-subject third instars of each species were collected as a subsample and stored in 70% ethanol for later study.
### Table 2. Sources of flies studied in this project

<table>
<thead>
<tr>
<th>Species</th>
<th>Source location</th>
<th>Source</th>
<th>Date collected/received</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Liopygia crassipalpis</em></td>
<td>Dr. David L. Delinger, Ohio State University</td>
<td>laboratory colony</td>
<td>4/18/2007</td>
</tr>
<tr>
<td><em>Neobelliera bullata</em></td>
<td>Dr. May Berenbaum, University of Illinois at Urbana-Champaign</td>
<td>laboratory colony</td>
<td>7/31/2007</td>
</tr>
<tr>
<td><em>Neobelliera bullata</em></td>
<td>Ames, Story Co., IA, USA</td>
<td>Jeff Alfred</td>
<td>7/23/2007</td>
</tr>
<tr>
<td><em>Wohlfahrtia vigil</em></td>
<td>Delta Junction, South East Fairbanks Census Area, AK</td>
<td>NVSL acc# 269212</td>
<td>7/28/2003</td>
</tr>
<tr>
<td><em>Wohlfahrtia vigil</em></td>
<td>Dr. Steven Krauth, Laboratory colony 1964</td>
<td>reference collection</td>
<td>5/2/2011</td>
</tr>
<tr>
<td><em>Neobelliera citellivora</em></td>
<td>5 Km east, 1 km south of Picture Butte, Alberta, CAN</td>
<td>Dr. Gail Michener</td>
<td>8/1/2002</td>
</tr>
<tr>
<td><em>Neobelliera citellivora</em></td>
<td>6 Km east, 1 km south of Picture Butte, Alberta, CAN</td>
<td>Dr. Gail Michener</td>
<td>8/1/2006</td>
</tr>
<tr>
<td><em>Neobelliera citellivora</em></td>
<td>7 Km east, 1 km south of Picture Butte, Alberta, CAN</td>
<td>Dr. Gail Michener</td>
<td>8/30/2007</td>
</tr>
<tr>
<td><em>Gigantotheca plinthopyga</em></td>
<td>Apache, Cochise Co., AZ, USA</td>
<td>Dr. Arnold Moorhouse</td>
<td>9/24/2007</td>
</tr>
</tbody>
</table>
Ten maggots of each species except *Wohlfahrtia vigil* (n=9), were used to gather all measurements and descriptive characteristics, other than the sclerotized features. In order to describe the sclerotized features, at least four cleared specimens were used for each species. Cuticular and other features of maggots for morphological study and description were observed using a dissecting microscope (10x – 200x) and a compound microscope (40x – 200x). Line drawings were made by tracing captured digital images photographed through one or the other of these two microscopes and manipulating the traced images to better represent a typical morphology for the particular species.

When designing this key, effort was made to use morphological features that were both easily described and easily identified, as well as characteristics that provided clear differentiation between character states. Although diagrams of cephalopharyngeal skeletons were included, they are more for verification purposes and not necessary for use of this key. Other standard features, such as anterior spiracles and posterior spiracular plates, were also used in this key. Also, many of the same or similar features as the ones thoroughly described by Sanjean (1957) and Ishijima (1967) were used.

One previously unused feature that was discovered during this study was the cuticular papillae. No previous reference by other authors to this feature’s use was found, but it proved to be important in the creation of the dichotomous key. Many of the species studied had, to varying degrees, minute papillae (or bumps) on the cuticular surface of the body. The cuticular spines found on some species seem
homologous to and derived from modified papillae. The presence, absence, or spine like modification of papillae, are character states used in this key.

Preliminary drawings were scanned, digitized, and enhanced, using Adobe Photoshop (Adobe Systems Inc., San Jose, CA). All measurements were made using the Leica application suite v3.7 (Leica Microsystems, Wetzlar, Germany), a Leica MZ9.5 dissecting microscope, and a Leica DFC490 camera with a 0.63x camera tube. Specimens for observation of sclerotized features were cleared overnight in a standard 180 μL ATL lysing buffer and 20 μL proteinase K (Qiagen, Germantown, MD) solution as part of DNA extraction procedures. In some cases, additional clearing was necessary; these specimens were subsequently soaked in heated 10% aqueous sodium hydroxide solution until they were sufficiently cleared. After clearing and rinsing with water and then 70% ethanol, excess cuticle was trimmed from the specimens, and the remaining cephalopharyngeal region and posterior spiracular plates were mounted in cavity slides using Hoyer’s mounting medium. Morphology of sclerotized features was observed, measured, and illustrated using the same methods and equipment as described for the cuticular features.

Voucher specimens were deposited in the reference collections at the USDA, NVSL, Parasitology Laboratory, and Iowa State University Department of Entomology in Ames, IA.
Results

Species descriptions

The following characteristics are shared to a greater or lesser degree by all sarcophagid myiasis species studied for this project: larvae of this group of sarcophagids are vermiform, generally cylindrical, tapering strongly anteriorly and slightly posteriorly, and yellowish-white in color, with 12 well-defined body segments (Fig. 1). They are generally larger than typical myiasis maggots, ranging in length from about 10 mm to almost 20 mm, with a mean length of 15.8 mm.

The two, bilaterally symmetrical, posterior spiracular plates reside in a caudal spiracular pit on somatic segment 12 (Fig. 1), the inside rim of which is ringed in fine hair-like spines. The sclerotized spiracular plates (Fig. 2) of the third-instar maggots studied are each composed of three ventrally convergent spiracular slits (Fig. 2) and a surrounding sub-circular peritreme (Fig. 2). Both the spiracular slits and the peritreme are variable in shape, thickness, and orientation, but these characteristics are usually relatively uniform within a particular species. The dorsal, outer rim of the spiracular pit (Fig. 3) displays two bilaterally symmetrical sets of three conical tubercles (Fig. 3), which may vary in shape and size in a diagnostically useful way. The space between outer and middle tubercles is approximately equal to the space between middle and inner tubercles, both of which gaps are less than the medial space between the inner tubercles. The outer tubercles are larger than either the middle or the inner tubercles. The ventral, outer rim of the spiracular pit also has two bi-symmetrical sets of three conical tubercles (Fig. 3). The inner set of these
tubercles is set slightly ventrad of the rim of the spiracular pit. Most often, the distance between the outer and middle tubercles is approximately equal to the distance between the middle and inner tubercles; both of these distances are greater than the medial distance between the inner tubercles. The middle tubercle is the largest, with the outer and inner tubercles being approximately the same size. Additionally, there is a minute symmetrical pair of tubercles medially on the inner, ventral surface of the rim of the spiracular pit. These tubercles may be small enough that they are obscured on some species by cuticular papillae, though they alternatively may be rather large. An anal protuberance (Fig. 3) projects from the posterior-ventral surface of somatic segment 12; this feature is less developed in some species. Two anal tubercles (Fig. 3) are situated distally on the anal protuberance, with an anal opening located between them. A patch of small, dark-tipped spines is present on the dorso-ventral cleft (Fig. 3) of the anal opening.

The surface of the somatic segments is almost always covered to a greater or lesser degree with minute cuticular papillae, or papillae modified into spines. Most species have a ring of 18 small tubercles divided into symmetrical triads, with one pair of triads each on the dorsal and ventral surfaces, and one set on each lateral surface, surrounding the approximate middle of each somatic segment, 5 – 11. Also, near the interface between the lateral and ventral surfaces, one tubercle on either side is larger than the rest. Midlateral welts are present on somatic segments 5 – 11 (Fig. 1), and creeping welts, on the ventral segmental margins, are variably developed.
The segmental margins are ringed both fore and aft in minute spines, papillae, or a combination of both.

A pair of multi-lobed, anterior spiracles (Fig. 4) are symmetrically present posterolaterally on either side of somatic segment two. A patch of small, dark-tipped cuticular spines is present ventrally on the margin between somatic segments one and two. Anteriorly, inside each maggot, is a group of associated sclerites, including three major sclerites and at least one minor sclerite, collectively called the cephalopharyngeal skeleton (Fig. 5). The anterior-most portion of the cephalopharyngeal skeleton, the paired mouth-hooks (mandibles) (Fig. 5), project through the oral opening from the venter of somatic segment one. Two antennomaxillary lobes reside anteriorly on somatic segment one, one on either side of the midline. On each lobe are two sclerotized rings, a larger ring circumscribing the maxillary palp, and a smaller ring that circumscribes the much reduced antenna.

The following descriptions are intended to supplement and highlight deviations from the previously described general description for this group of maggots. The species are again discussed in alphabetic order.

**Gigantotheca plinthopyga** (Figures 6 A, B, & C)

These maggots range in length from 16.3 to 18.7 mm, with a mean length of 17.6 mm, and a widest point from 3.4 to 4.2 mm, with a mean width of 4.0 mm. Tubercles surrounding the spiracular pit are well defined in this species, as are the anal protuberance and the tubercles that originate from it. The peritremes surrounding the
posterior spiracular slits (Fig. 6B) are the thinnest of all studied species. These peritremes are approximately circular, with almost the entire bottom open (i.e., discontinuous), and a strong, angular bend in the inside margin. All three spiracular slits are nearly straight and convergent ventrally to the opening.

Dorsally and laterally, segments 5 - 12 are completely covered by minute papillae, which continue into the spiracular pit, appear in patches posteriorly on the dorsal and lateral surfaces of segments 3 and 4, and present as spines on the ventral, segmental margins of all segments. Intrasegmental tubercles are well developed, and especially so ventrally, although they may be obscured dorsally and laterally by the cuticular papillae. The lateral welts and creeping welts are evident but only slightly protruding. The creeping welts alone are also covered with minute, dark-tipped cuticular spines.

Each anterior spiracle (Fig. 6C) has 10 – 12 fingers in a single, straight row, and a surrounding transparent membrane that may be evident only when viewed on a compound microscope after the specimen has been cleared.

**Liopygia argyroostoma** (Figures 7 A, B, & C)

These maggots range in length from 14.4 to 17.5 mm, with a mean length of 16.2 mm, and a widest point from 3.2 to 3.8 mm, with a mean width of 3.6 mm. Tubercles surrounding the spiracular pit are well defined and narrow in this species. The anal protuberance and the tubercles that originate from it are both well defined. The peritremes (Fig. 7B) are approximately ovate, with the bottom mostly closed. All
three spiracular slits are curved and convergent to the ventral continuation of the peritreme.

Dorsally and laterally, the margins of segments 5 - 12 are covered by minute spines, but the remainder of segment 12 only is covered with minute papillae that continue into the spiracular pit. The rings of intrasegmental tubercles are poorly developed and may be not visible. Lateral welts and creeping welts are evident but only slightly protruding.

Each anterior spiracle (Fig. 7C) has 13 – 15 fingers on multiple planes.

*Liopygia crassipalpis* (Figures 8 A, B, & C)

These maggots range in length from 16.3 to 18.5 mm, with a mean length of 16.9 mm, and a widest point from 3.7 to 4.5 mm, with a mean width of 4.1 mm. Tubercles surrounding the spiracular pit are small, but well defined in this species; the anal protuberance and the tubercles that originate from it are also well developed. The peritremes surrounding the spiracular slits (Fig. 8B) are almost circular, but discontinuous ventro-medially. The outside and middle spiracular slits are nearly straight, the inside spiracular slit is slightly curved, and all three slits are convergent to the ventral continuation of the peritreme.

Dorsally and ventrally, segments 5-12 are covered by papillae, which on segments 7-12 are more pronounced and spine-like. Papillae also appear laterally on segments 5-12, in patches posteriorly on the dorsal and lateral surfaces of segment 4, and present as spines around all segmental margins. Rings of intrasegmental
tubercles, if present, are poorly developed and obscured by the cuticular papillae. The lateral welts and creeping welts are evident but only slightly protruding.

Each anterior spiracle (Fig. 8C) has 10 – 14 fingers in multiple planes with varying lengths.

**Neobellieria bullata** (Figures 9 A, B, & C)

These maggots range in length from 15.7 to 17.8 mm, with a mean length of 16.6 mm, and a widest point from 3.6 to 3.9 mm, with a mean width of 3.8 mm. Tubercles surrounding the spiracular pit are well defined in this species, as are the anal protuberance and the tubercles that originate from it. Peritremes of the spiracular plate (Fig. 9B) are approximately semi-circular, with almost the entire bottom open. All three spiracular slits are slightly curved and convergent to the ventral opening.

Dorsally, ventrally, and laterally, segments 5 – 12 and the majority of segments 3 and 4 are covered by minute papillae, which continue into the spiracular pit on segment 12 and present as minute spines on all segmental margins. Rings of intrasegmental tubercles are well developed, although they are somewhat obscured by the cuticular papillae. The dorsal welts, lateral welts, and creeping welts are all well developed and contiguous, forming a swollen ring at the segmental margins. This ring is covered with a mixture of papillae and poorly developed spines.

Each anterior spiracle (Fig. 9C) has 18 – 20 fingers in a single, straight row, and a surrounding transparent membrane that may be evident only when viewed on a compound microscope after the specimen has been cleared.
**Neobellieria citellivora** (Figures 10; 11 A, B, & C)

These maggots range in length from 13.6 to 16.4 mm, with a mean length of 14.7 mm, and a widest point from 3.0 to 3.5 mm, with a mean width of 3.2 mm. Tubercles surrounding the spiracular pit are well defined, radially symmetric, and low, with gently rising sides. The anal protuberance (Fig. 11) is poorly developed and often not visible. The anal tubercles are well developed and often point posteriorly. The peritremes surrounding the spiracular slits (Fig. 10B) are variably shaped, with rounded angles, and are nearly closed. All three spiracular slits are slightly curved and convergent to the ventral continuation of the peritreme.

Only segment 12 is covered by minute papillae, which may be sparse dorsally and continue into the spiracular pit. Other segments may also have a few sparse papillae present. Rings of intrasegmental tubercles are undeveloped, and if visible, present as very slight bumps. The dorsal welts, lateral welts and creeping welts are well developed and contiguous, forming raised rings at all segmental margins that are covered in dark-tipped spines.

Each anterior spiracle (Fig. 10C) has 8 – 10 fingers in multiple planes.

**Wohlfahrtia vigil** (Figures 12; 13 A, B, & C)

These maggots range in length from 10.3 to 16.1 mm, with a mean length of 13.2 mm, and a widest point from 2.4 to 4.1 mm, with a mean width of 3.2 mm. Tubercles surrounding the spiracular pit are broad, radially symmetric, and shallow, with gently rising sides. The anal protuberance (Fig. 13) is well developed. The anal tubercles
are well developed and bulbous. The peritremes surrounding the spiracular slits (Fig. 12B) are generally round and completely open at the bottom. All three spiracular slits are nearly straight and ventrally convergent to the peritreme opening.

The body surface is covered in a reticulate pattern of sculpted grooves. Rings of intrasegmental tubercles are well developed. The dorsal welts and lateral welts are pronounced and covered in papillae, some of which are modified into spines. Creeping welts are well developed and present as three veruncles (Fig. 14). The combination of all the swollen welts gives this maggot a general rough and warty appearance.

Each anterior spiracle (Fig. 12C) has 8 – 10 fingers in multiple planes.

**Key to third-instar maggots of myiasis causing Sarcophagidae**

Note that many important diagnostic cuticular features may be obscured by fluids on the maggot surface. It is therefore important to remove the specimens from any liquid and completely dry the surface of all maggots before attempting to use this key for identification. Some cuticular features are more easily viewed in profile.

1. Laterally, somatic segments 5 – 11 fully covered with cuticular papillae that also may be present on segments 3, 4, and/or 12 ........................................ 2

   Lateral cuticular papillae on segmental margins only, as sparse patches on welts only, or not present ................................................................. 4
2. Anterior spiracles with 18 – 20 fingers (Fig. 9C)  
   Neobellieria bullata

   Anterior spiracles with 10 – 14 fingers (Figs. 6C & 8C)  
   3

3. Segments 5 – 11 with ventral tubercles presenting as a pair of well-developed bilateral triads (Fig. 15); peritreme of spiracular plate extremely thin and angular, with the entire bottom open; posterior spiracular slits nearly straight (Fig. 6B)
   Gigantotheca plinthopyga

   Ventral tubercles, if discernible, presenting as a pair of poorly defined, blister-like bilateral triads; peritreme of spiracular plate thick and circular, with bottom nearly closed; inside spiracular slit curved inward towards center slit (Fig. 8B)
   Liopygia crassipalpis

4. Welts on segmental margins and creeping welts poorly differentiated; tubercles surrounding spiracular pit fine and pointed  
   Liopygia argyrostoma

   Welts on segmental margins well developed; creeping welts prominent and have three distinct raised features (Figs. 16 & 17); tubercles surrounding spiracular pit wide and blunt tipped (Figs. 10 & 12)  
   5
5. Creeping welts have three distinct bumps with the outer swellings connected with an anterior bridge (Figs. 16 & 18); papillae on segmental margins differentiated/modified into spines, dark tipped and continued to ventral surface; anal protuberance poorly developed, but with well-developed anal tubercles (Fig. 11) ..........................  Neobellieria citellivora

Creeping welts present as three distinct independent swellings (Figs. 14 & 17); papillae on segmental margins differentiated/modified into minute spines without dark tips; anal protuberance well developed (Fig. 13) .............  Wohlfahrtia vigil
Figure 1. Lateral view of a typical sarcophagid maggot.

Figure 2. A typical posterior spiracular plate.
Figure 3. Dorsal view of a typical segment 12

Figure 4. Anterior spiracle of a typical maggot
Figure 5. A typical cephalopharyngeal skeleton
Figure 6. *Gigantotheca plinthopyga*: A. cephalopharyngeal skeleton, scale = 2 mm; B. anterior spiracle, scale = 1 mm; C. posterior spiracular plate, scale = 1 mm
Figure 7. *Liopygia argyrostroma*: A. cephalopharyngeal skeleton, scale = 2 mm; B. anterior spiracle, scale = 1 mm; C. posterior spiracular plate, scale = 1 mm
Figure 8. *Liopygia crassipalpis*: A. cephalopharyngeal skeleton, scale = 2 mm; B. anterior spiracle, scale = 1 mm; C. posterior spiracular plate, scale = 1 mm
Figure 9. *Neobellieria bullata*: A. cephalopharyngeal skeleton, scale = 2 mm; B. anterior spiracle, scale = 1 mm; C. posterior spiracular plate, scale = 1 mm
Figure 10. *Neobellieria citellivora*: A. cephalopharyngeal skeleton, scale = 2 mm; B. anterior spiracle, scale = 1 mm; C. posterior spiracular plate, scale = 1 mm

Figure 11. Dorsal view of segment 12 of *Neobellieria citellivora*, scale = 1 mm
Figure 12. *Wohlfahrtia vigil*: A. cephalopharyngeal skeleton, scale = 2 mm; B. anterior spiracle, scale = 1 mm; C. posterior spiracular plate, scale = 1 mm

Figure 13. Dorsal view of segment 12 of *Wohlfahrtia vigil*, scale = 1 mm
Figure 14. Ventral view of segments 7, 8, and 9 of *Wohlfahrtia vigil*, scale = 1mm.

Figure 15. Ventral view of segments 7, 8, and 9 of *Gigantotheca plinthopyga*, scale = 1mm.
Figure 16. Posterior profile of *Neobellieria citellivora* ventral segment 8, scale = 1mm.

Figure 17. Posterior profile of *Wohlfahertia vigil* ventral segment 8, scale = 1mm.

Figure 18. Ventral view of segments 7 and 8 of *Neobellieria citellivora*, scale = 1mm.
Discussion

Evidence suggests it is important to consider sarcophagids in North American myiasis, furthermore, some species may have agricultural benefits. Several species (including some discussed in this study) are parasitic on grasshoppers and other potential arthropod pests (Rees 1973) and mollusks (Barker 2004). Also, the scavenging habits of many species of sarcophagid help with the breakdown of carcasses and feces. Among myiasis flies, the commonness and widespread distributions of *L. argyrostoma*, *L. crassipalpis*, and *N. bullata* would suggest that they may be of importance in some locations. Also, *W. vigil* is a known pest in the fur industry, but the simple techniques used to prevent infestation of newborn animals have reduced current losses to this fly.

Even though *B. africa* has a near cosmopolitan distribution, it may be less common than dogma would suggest, and the inability to acquire specimens of this species for study leaves a void in the identification key and the associated descriptions. Unfortunately, without verifiable identifications in the literature and considering the nomenclatural flexibility of *S. haemorrhoidalis*, a major synonym of *B. africa*, the true importance of this species as a myiasis fly is not known.

In addition to several earlier myiasis literature reports, collections made from road kill and beef liver bait in Arizona (as part of the the present study) almost always contained or were wholly composed of *G. plinthopyga* maggots. This evidence suggests that *G. plinthopyga* probably is an important sarcophagid species in myiasis in the southwestern states.
In her 3-year study, Michener (1993) found that almost 9% of juvenile and 1% of adult Richardson’s ground squirrels were infested by *N. citellivora* larvae. Many of these infestations led to death of the hosts. The ecological and agricultural importance of these ground squirrels and the degree of impact these deaths have on the species are not discernible from literature reports, but in their range, these ground squirrels are a food source for several predators (Michener 2010).

One cannot discount the possibility that other species of sarcophagid maggots, particularly those on my initial list of suspects (Appendix I), could or do occasionally cause myiasis. Any species that feeds on carrion or feces could easily move from a necrotic wound or fouled hair to the living tissues of a host animal. Even so, specimens identified at the NVSL and records in the literature more prominently involve the species of note listed in Table 1.

Although this key does not include *B. africa*, it is still useful in identification of the important species it does cover, and it will allow and facilitate identification of most third-instar sarcophagid myiasis maggots encountered in North America north of Mexico. Care should be taken, however, to not regard this key as all-encompassing. Maggots that do not match the provided descriptions may prove to be a species not in the purview of this study.
Chapter 3. Molecular identification of myiasis-causing sarcophagids of North America north of Mexico

Literature Review

DNA is actively used in species-level identification of organisms in many fields, including forensic science (Chen et al. 2004, Guo et al. 2011), animal health (Otranto and Stevens 2002, Armstrong and Ball 2005), and the identification of new species (Herbert et al. 2004). Although identification of many species is easily done by morphological means with dichotomous keys, morphology alone may not allow identification of immature life stages, cryptic species, or damaged specimens (Armstrong and Ball 2005, Dawnay et al. 2007). DNA identification techniques overcome such issues because they allow accurate species-level identification using any life stage or body fragment (Savolainen 2005, Draber-mońko et al. 2009).

The use of insects and of DNA in forensic investigations is nothing new, and the joining of these two lines of evidence gathering has been widely accepted. In fact, there is a body of literature on use of DNA for identification of forensically important Diptera (Chen et al. 2004, Guo et al. 2011, and many others). One of the most common uses of maggots in forensic investigations is to calculate the postmortem interval (PMI), i.e., the time between the death of a crime subject and when the body is found, based on the developmental state of maggots collected from the body. Several factors must be known to accurately calculate a PMI, one of which is the characteristic development time of the maggot species found (Byrd and Castner...
To know what standard development time to use for calculations to be accurate and precise, first it is necessary that proper species identification is made (Zehner et al. 2004).

One aspect of the animal health field is early identification of exotic and potentially invasive pathogenic species (Armstrong and Ball 2005). Our ability to make such identifications can be hindered by lack of knowledge about exotic species and compromised condition of submitted specimens (Armstrong and Ball 2005). Myiasis is an important animal health concern, and accurate identification of some immature Diptera is difficult or impossible due to lack of adequate identification keys and descriptions.

DNA barcoding will be useful and effective in species identification only with access to a large database of known species and their associated barcodes (Savolainen et al. 2005, Guo et al. 2011). To assure value and viability, all such databases will require, at a minimum, that initial identifications of reference specimens are correct. Misidentifications of reference source specimens have occurred and resulted in incorrect identifications of unknown specimens. In at least one case, the error was discovered and corrected by the submitters of the unknown specimen (Wells 2002).

Currently, the Barcode of Life Web site (http://boldsystems.org) houses about 114,000 different species barcodes, with the long-term goal of barcoding all of the estimated 10 million species on Earth (Savolainen et al. 2005). The cytochrome oxidase subunit I (COI) gene is currently the most commonly used region for barcoding (Herbert et al. 2003, Roe and Sperling 2006, Guo et al. 2011).
Methods and Procedures

DNA extraction

Names and sources for the flies and maggots used in this study are listed in Table 2.

DNA was extracted from one-half of a maggot each in testing *G. plinthopyga*, *L. argyrostoma*, and *L. crassipalpis*, and from one thorax each of adult *N. bullata* and *N. citellivora*. Each specimen was cut with a new razor blade. DNA was extracted using the insect protocols for the DNeasy® Blood and Tissue Kit (Qiagen, Germantown, MD), with four modifications. Because the sclerotized features of the maggots processed were used for morphological study, those samples were not ground-up during extraction. After addition of ATL buffer and proteinase K, samples were incubated overnight at about 56°C; another 20 μL of proteinase K was added, and the mixture was incubated for about 6 hr more. A second wash in 500 μL of buffer AW2 was performed immediately after the first AW2 wash and by following the same technique. Extracted DNA was eluted into 60 μL AE buffer, which was incubated for 5 min instead of 1 min, before centrifugation.

Initial attempts at polymerase chain reaction (PCR) to isolate the COI gene from flies using COI primers of Zehner et al. (2004) yielded product for only three of the six species processed. Attempts to refine the protocol for these reactions with this set of primers were unsuccessful, and primers from Simon et al. (1994) were used instead. DNA was not successfully isolated from maggots of *Wohlfahrtia vigil* and *Neobellieria citellivora*. For these two species, adult fly specimens were used for
DNA extraction and sequencing. A sequence was obtained from the adult *N. citellivora* DNA but not from the adult *W. vigil*.

**DNA quantification**

Quantification of DNA was performed on a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA) before each of the two PCR reactions.

**PCR**

PCR was used to isolate the COI gene segment to be sequenced using primers C1-J-1751 and C1-N-2191 (Simon et al. 1994).

C1-J-1751 (5’ GGAGCTCCTGAGACATAGCATTCCC 3’)
C1-N-2191 (5’ CCCGGTAAAATATAAAATAACTTC 3’)

Thermocycling parameters were a modification of those used in Sperling et al. (1994). Initial incubation was at 94°C for 3 min, followed by an annealing phase at 45°C for 1.5 min, and then an extension phase at 72°C for 1.5 min. All of this was followed by 34 cycles of 94°C for 1 minute, 45°C for 1.5 min, and 72°C for 1.5 min. The final extension lasted 5 min.

After the products from these initial PCR reactions were quantified, each was diluted to about 14 μg/μL, as instructed in the sequencing protocols. Sequence PCR reactions were set-up using only the C1-J-1751 primer and using the thermocycling parameters provided by the manufacturer (Beckman Coulter, Fullerton, CA). Four replicates of a single sample were run for each fly species.
Sequencing

DNA sequencing was performed on a CEQ™ 8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA), following the manufacturer's protocol.

Sequence processing

Usable DNA sequence from reactions that produced them for each fly species were compared against the other sequences identified from that same species to create a consensus sequence for each using SeqMan Pro™ (DNASTAR Inc., Madison, WI). These consensus sequences were used for comparison to on-line public databases of DNA sequences (e.g., Barcode of Life Database [BOLD]). The only replicate that provided a sequence not used to produce a consensus sequence was one from *N. citellivora* that was 559 bases long, 72 bases longer than an expected sequence should be. This sequence did not align well with the three other replicate sequences obtained for this species. Given the anomalous size of this replicate and its poor alignment, it was assumed to be a spurious sequence and was not used for further study.

Results

Initial sequence results for *N. bullata* DNA showed a 100% match to the *L. argyrostroma* sequence. This caused me to doubt my identification of the maggot specimen used in this initial sequencing. So, new sequencing was performed for *N. bullata*, using DNA extracted from an adult male fly, a pupa, and a different maggot specimen. Thus, sequences were collected from three life stages, but the analyzed
sequences from the pupa were much longer than they should have been and were not used. DNA sequences from the maggots and the adults were similar to each other, but the consensus sequence of the adult sample was a slightly better match to the existing BOLD entries than the maggot consensus sequence, so my final analysis used the adult sample sequence for *N. bullata*.

The consensus DNA sequences for each fly species (Appendix 2) were entered into the BOLD identification engine (www.boldsystems.org), and a matching search of the species-level barcode records was performed. The *Gigantotheca plinthopyga* sequence had a 100% match to the database entry for the same species, with the next closest entry being *N. cooleyi*, with a 93.19% match. There is a BOLD entry for a sample designated “sarcJanzen01 Janzen15” with a 99.74% match to my sequence, and additional information for this sequence on the BOLD Web site listed the source of this sequence as a fly caught in Costa Rica. Because *G. plinthopyga* occurs in Costa Rica (Pape 1996), and the sequence is such a close match, it is likely that this sequence is also from a *G. plinthopyga* specimen. My *Liopygia argyrostroma* sequence has a 100% match to the database entry of the same species, and the next closest match is *L. ruficornis*, with a 93.64% match. The *Liopygia crassipalpis* sequence had a 100% match to the database entries of the same species, with the next closest match being *L. ruficornis*, with a 95.77% match. The *Neobellieria bullata* sequence had a 97.55% match to the database entries of the same species, with the next closest match being *N. cooleyi*, with a 96.08% match. The sequence for *N. citellivora* most closely matched the BOLD sequence for
*N. cooleyi* (96.57%). Because a sequence for *N. citellivora* has not been entered in the database, it is not surprising that there was no exact match for this species.

Finally, the consensus DNA sequences were compared to each other (Table 3), using the BLAST (Basic Local Alignment Search Tool) nucleotide comparison tool (http://blast.ncbi.nlm.nih.gov) optimized for highly similar sequences. All reported matches were between 90 – 92% similarity to each other, with e-values ranging from $3 \times 10^{-73}$ to $3 \times 10^{-154}$, except the match between *N. citellivora* and *N. bullata*, which was 95% with an e-value of 0.
Table 3. Percent match and e-value of that match for COI consensus sequences of studied sarcophagids (see text for explanation of e-value)

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Discussion

The literature implicates 19 species of sarcophagids in myiasis in North America north of Mexico, but of the 19 species, there are valid data to support seven being important to myiasis in the stated region. DNA barcoding was carried out on six of the seven, with usable sequences obtained from five of those. Most of the seven species have existing barcode sequence data available (Wells et al. 2001), including *B. africa* (Zehner et al. 2004), *G. plinthopyga* (Stamper 2008), *L. argyrostroma* (Draber-Mońko et al. 2009), *L. crassipalpis* (Tan et al. 2010), *N. bullata* (Stamper 2008), and *W. vigil* (Wells et al. 2001).

Consensus DNA sequences for five of the sarcophagid species of interest were acquired, and when tested against the BOLD, all species had a 100% sequence match to the existing database entries for the same species, except *N. bullata* (97.55% match) and *N. citellivora* (for which there were no extant sequences to compare).

Byrd and Castner (2001) found that carrion flies could be differentiated to species, using DNA sequences about 300 bp long. However, longer sequences might offer the possibility of greater variance between species sequences and thus may be preferable. To this end, different primers that result in longer sequences might be studied and applied. In addition, the range of applicability for this study might be broadened by sequencing the DNA from other sarcophagid species that may be implicated in myiasis, especially those listed in Appendix 1.
DNA sequence was not obtained from either immature or adult *W. vigil*. Wells et al. (2001) did obtain *W. vigil* DNA sequences, but these authors did not state specifically which primers they used to achieve their results, and perhaps a different set of primers would have produced better results.

Of the six species of sarcophagid for which samples were obtained, sufficient, diverse, morphological features were found that allowed for the creation of a dichotomous key that can be used to identify the most significant myiasis-causing sarcophagids in North America north of Mexico. Also, the methods outlined in this report for DNA sequencing of a portion of the COI gene proved effective for molecular identification of four of the five species whose sequence data was acquired. Because many other sarcophagid COI sequences have been placed in public databases by Wells et al. (2001), Tan et al. (2010), and others, the results of this report should coordinate to allow for the identification of species for which it does not have sequence data.
References Cited


James, M. T. 1944. Two erroneous records in American literature of the causative agents of myiasis. J. Parasitol. 30: 273-274.


Knowlton, G. F., and D. G. Hall. 1941. Flesh fly kills kit mink. J. Econ. Entomol. 34: 216.


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Summaries of previously reported Sarcophaegidae myiasis cases.

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Appendix 2.

Consensus DNA sequences of sarcophagid species studied

**Gigantotheca plinthopyga** – 413bp (GenBank acc# JN873916)

CTTTAACATT ATTACTAGTA AGTAGTATAG TAGAAAATGG AGCTGGAACA GGTTGAACCTG
TTTACCCCTCC TTTATCTTCT AATATTGCCA ATGGAGGAGC ATCTGGTTGAT TTAGCAATT
TCTCTCTTCA CTTAGGTGGA ATTTACCTTA TTTTAGAGGC AGTAAATTTTT ATTACTACAG
TAATATAATACAGATCTAAC GGTATTTATTGTGACTGAAT ACCTTATTTTT GTTTAGATCTG
TAGATAATTACTGCTTTATTATAATTCTTGTACTTCTGTGAGAGCTTCTGTTACATTTCTTAACATTTCTAATTACACCTTA TTCTGATCTC TTGGACATCC TGAAGTTTAT ATT

**Liopygia argyrostroma** – 434bp (GenBank acc# JN873917)

GACTTTTACCTCCTGCAACATACATTACTAC TAGTAGTAGA TAGTAGGAGGA AATGGAGCTG
GAACAGATGGAACTGTTTTAT CCTCCTTATATGACTGAAT CATCTACATG GAAGCTTCTG
TTGATCTAGCTATTTTTCTTCTTCCTTAGCTGGAAATTTCC CTGGAATTTG TTCAATTTTTA GAGCAGTAA
ATTTTATCACGAAATTTACTGACTGAT ATCTGGTTGATTTTTAT ATTTTTTTTTGT CATCCTGAAGTTTAT ATT
**Liopygia crassipalpis** – 403bp (GenBank acc# JN873918)

GCTTCTAGTA AGTAGTATAG TAGAAAAATGG AGCTGGAACG GGGTGAACCTG TTTACCCTCC
TTTAGGAGGTT ATGGAAGAATG CTCTGGAT TTAGCATTTT TTTCTCTACA
TTTAGGAGGTT ATGGAAGAATG CTCTGGAT TTAGCATTTT TTTCTCTACA
ACGATCTACA GGAATTACCT TTGATGAAAT ACCTTTATTT GTTTGATCTG TAGTAATTAC
AGCCCTACTTT TTTACCGGT ACTTGAGGGA ATCTATACCTA TATTATAAC
TGACGCCTACT TTACCTTTAT CTTTACGAGA GGAGGAGATC CTATTTTATA
CCAACACCTA TTTTGATTTT TCGTACCC TGAAGTTTATT

**Neobellieria bullata** – 409bp (GenBank acc# JN873919)

ACTTCTAGTA AGTAGTATAG TAGAAAAACGG AGCTGGAACA GGATGAACCTG TTTACCCTCC
TTATCTTTCT AACATCGCCC ACGGAGGAGC TTCTGGAT TTAGCATTTT TTTCCCTACA
TTTAGGAGGTT ATGGAAGAATG CTCTGGAT TTAGCATTTT TTTCTCTACA
ACGATCTACA GGTATACAT TTGATGAAAT ACCTTTATTT GTTTGATCTG TAGTAATTAC
AGCTTTACCT TTACTTTTCT CCCTACCTGT ACTTGAGGGA ATCTATACCTA TATTATAAC
TGATCGAAAT ATTAATACCT TTTCTTTGAA CCCAGCAGGA GGAGGAGATC CTATTTTATA
CCAACATTTA TTTTGATTCT TCGTACCC TGAAGTTTAT
Neobelliera citellivora – 409bp (GenBank acc# JN873920)

ACTTCTAGTA AGAAGTATAG TAGAAAAACGG AGCTGGGACA GGATGAACCTG TTTACCCTCC
TTTATCATCT AATATCGCTC ACGGCGGAGC TTCTGTGGAT TTAGCTATTTC TTTCCCTTCA
CTTAGCAGGA ATTTCTTCAAA TTTTAGGAGC AGTAAATTTT ATTACTACAG TTATTAATAT
ACGATCTACA GGTATTACAT TTGATCGAAT ACCTTTATTT GTTTGATCTG TAGTAATTAC
AGCTTTACTT TTACTTTTTT CCTTGCTGGT ACTTGCTGGA GCAATTACTA TATTATTAAC
TGATCGAAAT ATTAATACCT CATTCCTTGAC CCCGCAGGAA GGAGAGATTC CAATTTTATA
CCAACATCTA TTTTGATTCT TTGGACACCC TGAAGTTTT ATTTTAATT
Acknowledgements

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