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RESEARCH ARTICLE

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An assessment of the accuracy of morphological techniques for identifying *Lucilia cuprina* and *Lucilia sericata* (Diptera: Calliphoridae)

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ABSTRACT

Aims: To assess the accuracy of the morphological identification of *Lucilia cuprina* and *Lucilia sericata* by using molecular analysis as a reference standard test, and to describe the seasonality of these species.

Methods: A convenience sample of *L. cuprina* and *L. sericata* flies was caught on eight farms from across New Zealand and stored at room temperature in 70% alcohol. They were first morphologically identified using published keys and then molecularly identified using primers to amplify the 28S rRNA region of the nuclear genome. The accuracy of the morphological identification was then estimated for each species using the molecular identification as a reference standard test. The correctness of the published keys was also tested by re-examining a sample of misidentified flies using enhanced magnification and photography.

Results: The accuracy of the morphological identification for *L. cuprina* was 0.66 (95% CI = 0.58–0.73) and for *L. sericata* was 0.7 (95% CI = 0.62–0.77). There was no evidence for a difference in accuracy between species (p = 0.56), and re-examination of the misidentified flies found no faults in the published keys. The study confirmed that *L. cuprina* has a longer season of activity than *L. sericata*.

Conclusions: These results emphasise the need to use molecular methods to confirm the identification of these species, especially when dealing with large, stored collections, rather than to rely on morphological identification alone.

Clinical relevance: Without accurate fly identification and knowledge of insecticide resistance status, effective control and prevention of flystrike in New Zealand could be handicapped.

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Lucilia cuprina; *Lucilia sericata*; flystrike; New Zealand; 28S rRNA region

Introduction

Flystrike has been estimated to affect 3–5% of the 26 million sheep currently farmed in New Zealand (Corner-Thomas *et al*. 2017), resulting in serious animal welfare and financial costs. Flystrike is defined as the infestation of the tissue of live vertebrates by larvae from the arthropod order Diptera that feed for varying periods of time on the hosts' tissue or bodily substances (Zumpt 1965). There are two species of *Lucilia* currently found in New Zealand that initiate the majority of cases of flystrike in sheep (Ullyett 1950; Heath and Bishop 2006): *Lucilia cuprina* (Wiedemann 1830) and *Lucilia sericata* (Meigen 1826).

Lucilia sericata is considered to have arrived in New Zealand in the late 1880s from Europe (Miller 1939), whereas *L. cuprina* only arrived in New Zealand in the late 1970s and took time to establish (Heath and Bishop 1995, 2006). There are two subspecies of *L. cuprina*: *Lucilia cuprina cuprina*, found in much of Asia and the northern tropical regions of Australia, and *Lucilia cuprina dorsalis*, which originates from Africa and is the dominant *L. cuprina* subspecies found in Australia and New Zealand (Bishop 1995; Stevens and Wall 1996; Wallman *et al*. 2005).

Historically, morphological keys have been extensively used to identify these flies (Dear 1986; Holloway 1991; Wallman 2001). However, accurate morphological identification suffers when specimens are either damaged or decomposed. Where this occurs, a variety of molecular tools may be employed to identify the species. For example, the nuclear gene encoding the 28S subunit of ribosomal RNA (28S rRNA) is highly conserved within the dipteran genera and can reliably distinguish both subspecies of *L. cuprina* from *L. sericata* (DeBry *et al*. 2010; Williams and Villet 2013).

Although both species can initiate flystrike there are important differences in their geographical distribution, preferred habitats, insecticide resistance (Wilson 1999; Waghorn *et al*. 2013), and where on the body they strike affected animals, which means

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accurate differentiation is still important. For *L. sericata*, flystrike is likely an extension of carrion feeding, especially when the carcase is in the open (Appleton 1993; Smith and Wall 1997), whereas *L. cuprina* are only rarely found on carrion (Waterhouse 1947; Dymock and Forgie 1993; Heath and Appleton 1999). This may have implications for evolving insecticide resistance, since by their feeding habits, *L. cuprina* will place themselves under more selection pressure than *L. sericata*, as carrion is usually free of chemicals. Furthermore, *L. cuprina* seems to predominate in breech strikes, whereas *L. sericata* affects other sites of the animal as well (A Heath[1], pers. comm.; French *et al*. 1995). This has implications for control strategies such as crutching. The likely effects of climate change on New Zealand will also have implications for the distribution of these two species (Heath 2021). *L. cuprina* is a sub-tropical species preferring hot, dry open conditions whereas *L. sericata* is a more temperate species, preferring cool, humid vegetated conditions (Waterhouse and Paramonov 1950). So, in areas of New Zealand that are projected to become hotter and drier, i.e. the north-east, *L. cuprina* (Wiedemann 1830) will predominate if sheep farming is still possible in these areas, whereas the west of both Islands is projected to become wetter, and we could see *L. sericata* (Meigen 1826) in ascendence (Heath 2021). Another consequence of global warming is that the flystrike season will likely start earlier in the year, and in New Zealand could potentially extend into winter (Tougeron *et al*. 2020). All these changes, in combination with others such as animal husbandry practices, will impact flystrike control and prevention at the farm level and, in order to advise their clients on targeted interventions, veterinarians will require accurate fly identification.

The research described in this publication was an appendage to a larger study that developed a predictive model for the seasonality of *Lucilia* spp. in New Zealand (Brett 2023). The aim of this supplementary study was to assess the accuracy of the morphological identification of *L. cuprina* and *L. sericata* results by using molecular analysis of the 28S rRNA gene as a reference standard test. Furthermore, using the molecular results, it was hoped to enhance knowledge of the seasonality of these two species.

Materials and methods

Specimen collection

Eight farms distributed across New Zealand were enrolled in the larger study (Figure 1). Farm selection was non-random and based on the willingness of farm owners and/or managers to be involved in this project. Flies were collected on each farm using three LuciTraps (Bugs for Bugs, Toowoomba, QLD, Australia) with an additional StickyTrap (EasyTrap Stickies for Flying Insect; Gubba, Auckland, NZ) placed on the lid between the entry holes of each LuciTrap. These were emptied once weekly. For a full description of the collection methodology, see Brett *et al*. (2024).

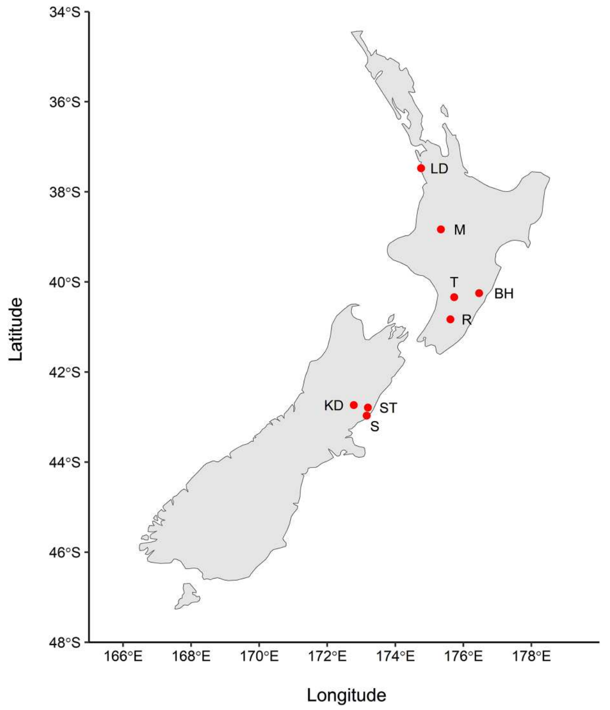

Figure 1. A map of New Zealand, where individual letters identify the location of the eight farms enrolled in a study assessing the accuracy of morphological techniques for identification of *Lucilia cuprina* and *Lucilia sericata*.

Morphological identification

Once collected, samples were stored in 70% alcohol at room temperature. Only intact specimens were selected for this comparative study. Identification was carried out using a Leika EZ4 Stereo Microscope with a zoom range from 0.8–3.5x (Leica Microsystems GmbH, Wetzlar, Germany) and was based on seven morphological characteristics (Table 1). These characteristics were not developed by the lead author but chosen from an examination of the literature regarding identification of *L. sericata* and *L. cuprina* adults from the Australasian region (Dear 1986; Holloway 1991; Wallman 2001). For each specimen, a combination of these seven characteristics was used to make the identification. Owing to the large numbers of Diptera collected, no attempt was made to separate the flies by sex, and differences in morphology of the genitalia were not used to

[1]A Heath; AgResearch Ltd, NZ.

Table 1. The seven morphological characteristics used for identification of *Lucilia cuprina* and *Lucilia sericata* in a study assessing the accuracy of morphological techniques for identification.

Characteristic	*L. cuprina*	*L. sericata*
Number of hairs below each inner vertical bristle	0–2	2–8
Number and location of hairs on the humeral callus near the base of the wing	0–4	6–8
Metasternal area	Bare	Setose (bristly)
Colour of fore femora	Metallic green	Metallic blue to black
Number of hairs on the edge of the notopleuron behind the posterior notopleural bristle	2–5	8–16
Length of bristles on scutulum	Dorsal bristles slightly shorter or equal to lateral hairs	Dorsal bristles much shorter than lateral hairs
Width of the frontal stripe	As wide as parafrontal plate	Twice as wide as the parafrontal plate

identify the species (Stevens *et al*. 2002; Williams and Villet 2014).

To check the accuracy of the morphological keys a small number of misidentified flies were retrospectively re-examined, when the molecular results were known, using enhanced magnification and photography. For this, a total of 12 dried pinned specimens of *L. cuprina* and *L. sericata* were chosen, which was considered an adequate sub-sample based on previous studies (Stevens *et al*. 2002; Williams and Villet 2014). Photographs of each specimen were taken using an Olympus SZX7 (Olympus Corp., Tokyo, Japan). To increase the depth of field of the images, multiple images were taken at different focal distances with a focus stacking method using the software Helicon Focus (Helicon Soft, Kharkiv, Ukraine). This software uses a pyramid smoothing function to compose the final detailed images, which were used to identify whether the species was *L. cuprina* or *L. sericata* using the keys in Table 1.

Although the intention was to use a random sample of flies by farm and month, irregular catches and fly damage meant a convenience sample of the best specimens available over the 10-month collection period was utilised instead. Up to four flies, two identified morphologically as *L. cuprina* and two identified as *L. sericata,* were selected each month, depending on fly availability, from each farm over the collection period. After morphological identification, these samples were then dried on a paper towel, placed on an insect pin, and stored in an entomological box at room temperature. The morphological identification was carried out by a single experienced person (PTJB).

Molecular identification

Two hind legs were removed from each fly using forceps and placed into a 1.5 mL microcentrifuge tube. The legs were then crushed using a micro-pestle for approximately 30 seconds. The remaining portion of the fly was retained for later reference.

A salting-out DNA extraction protocol was used (Sunnucks and Hales 1996). The crushed samples were incubated overnight at 55°C with 600 µL of TNES buffer (50 mM Tris, 400 mM NaCl, 20 mM EDTA, 0.5% v/v sodium dodecyl sulfate) with 10 µL of Proteinase K (20 mg/mL) (Ambion; Thermofisher, Waltham, MA, USA). They were then placed on a heat block at 100°C for 5 minutes to denature the Proteinase K. The DNA was precipitated with 85 µL of 5 M NaCl and one volume of 100% ethanol overnight at −20°C. Following precipitation, the DNA was washed twice with 70% ethanol and dried. The air-dried DNA was resuspended in 20 µL of nuclease-free water at 4°C.

The 28S rRNA region in the nuclear genome was amplified in a total reaction volume of 20 µL containing 2 µL of template DNA, 1x Solis Biodyne HotFirePol (Solis Biodyne, Tartu, Estonia), 0.2 µM of each primer (Integrated DNA Technologies, Coralville, IA, USA), made to the final volume with nuclease-free water. The primers were from Stevens *et al*. (2002): 28S rRNA_F 5′-GAG GGA AAG TTG AA AGA AC-3′ and 28S rRNA_R 5′-GTT AGA CTC CTT GGT CCG TG-3′. A touchdown PCR protocol was carried out in a Mastercycler Nexus GX2 (Eppendorf, Hamburg, Germany) in 4 stages: an initial hold at 95°C for 15 minutes; 10 cycles of 95°C for 30 seconds, 60°C (decreasing 1°C per cycle) for 30 seconds, and 72°C for 30 seconds; then 40 cycles of 95°C for 30 seconds, 50°C for 30 seconds, and 72°C for 1 minute; then a final extension for 7 minutes at 72°C after which the reaction was held at 10°C. Samples were stored at 4°C until visualisation.

To assess the PCR results, the products were electrophoresed through 1% agarose gels (Bioline, London, UK), in 1 X TAE (Tris-acetate-EDTA: 40 mM Tris, 20 mM acetate and 1 mM EDTA) stained with 5 mM of Redsafe (iNtRON, Seongnam, Republic of Korea). Amplicons of the appropriate size were extracted from the gel and submitted to Massey Genome Service (Massey University, Palmerston North, NZ) for bidirectional Sanger sequencing.

Sequence analysis

Sequences were visually assessed for quality, and the consensus sequences were pairwise aligned using Geneious (version 10.2.6, BioMatters, Auckland, NZ). The published reference sequences for 28S rRNA of *L. cuprina* (AJ417709.1) and *L. sericata* (FJ650535.1) were used to confirm the morphological identification of either species. For sequences that did not match either of the reference sequences, a search was made on the National Centre for Biotechnology Information nucleotide database using BLAST (Basic Local Alignment Search Tool). The following sequences were hereafter used as reference sequences: *Chrysomya rufifacies* (Diptera: Calliphoridae; Macquart 1842) (JN014936) and *Pales pavida* (Diptera: Tachinidae; Meigen, 1824) (AB466084.1).

Statistical analysis

The accuracy of morphological identification, with 95% CI, was estimated for each species using the following formula:

$$\text{Accuracy} = (\text{TP} + \text{TN})/(\text{TP} + \text{TN} + \text{FP} + \text{FN})$$

where TP = true positive, TN = true negative, FP = false positive and FN = false negative. The 95% CI was calculated using the Wilson score interval, with the continuity correction method. A χ^2 test was performed to determine if there was a difference between the identification accuracy for *L. cuprina* and *L. sericata*. All analyses were carried out in R v4.4.2 (R Core Team 2021, R Foundation for Statistical Computing, Vienna, Austria). The results of the molecular identification by month were plotted for examination.

Results

Between September 2018 and June 2019, a total of 60,428 Diptera were caught across the eight farms, of which 2,752 (4.6%) were morphologically identified as *L. sericata*, and 5,680 (9.4%) were morphologically identified as *L. cuprina*. The individual demographic and catch data for the eight farms are summarised in Table 2. The total Diptera catches were higher on the South Island farms (KD, ST, and S).

Estimation of the accuracy of morphological identification

From these collections, a total of 197 flies were selected for the study, of which 124/197 (62.9%) were morphologically identified as *L. cuprina* and 73/197 (37.1%) were identified as *L. sericata*. However, for 32/197 (16.2%) of the selected flies, the molecular identification was not completed, because the 28S rRNA region could not be successfully sequenced. This left 165 flies which were successfully identified using molecular techniques, of which 105 had originally been morphologically identified as *L. cuprina* and 60 as *L. sericata*. The full results are shown in Table 3.

The accuracy of the morphological identification for *L. cuprina* was 0.66 (95% CI = 0.58–0.73) and for *L. sericata* was 0.70 (95% CI = 0.62–0.77). The χ^2 test found no support for a difference in the accuracy of morphological identification between fly species (p = 0.56).

Figure 2 shows the distribution of fly species identified using molecular identification by month across the eight farms. Both species of fly were present across all eight farms from November to April, whereas only *L. cuprina* was present in October and May. The accuracy of morphological identification for both species was poorest in December.

The results from morphological re-examination of a sample of misidentified flies after molecular identification showed that the misidentification was likely human error and not due to a fault in the published keys. Furthermore, no single characteristic was identified that led to the incorrect identification of these species. Figure 3 shows a comparison of the central occipital region of *L. cuprina* and *L. sericata* and Figure 4 shows

Table 2. Farm characteristics, number of Diptera collected, and the number morphologically identified as *Lucilia* genus, *Lucilia sericata* or *Lucilia cuprina* on each farm enrolled in a study assessing the accuracy of morphological techniques for identification.

Farm	Region	Altitude (m)	Size (ha)	Main sheep breed	Total Diptera collected	*L. sericata*	*L. cuprina*	Total *Lucilia* genus (%)
T	Manawatū	80–250	476	Romney	7,759	142	337	479 (6.2%)
R	Wairarapa	180–250	686	Romney	6,246	207	392	599 (9.6%)
BH	Hawkes Bay	100–420	1,300	Perendale	7,605	120	227	347 (4.6%)
M	Ruapehu	280–450	1,750	Romney	2,792	358	758	1,116 (40.0%)
LD	Waikato	0–200	1,500	Romney	7,058	212	454	666 (9.4%)
KD	Canterbury	280–450	3,219	Romney	9,368	552	1,023	1,575 (16.8%)
ST	Canterbury	160–280	1,800	Romney	8,135	705	1,520	2,225 (27.4%)
S	Canterbury	0–100	3,000	Romney	11,465	476	1,066	1,542 (13.4%)

Table 3. Comparison of the identification results for *Lucilia cuprina* and *Lucilia sericata* using morphology or using the 28S rRNA gene as the reference standard test in a study assessing the accuracy of morphological techniques for identification.

	Molecular identification					
Morphological identification	*L. cuprina*	*L. sericata*	*Chrysomya rufifacies*	*Pales pavida*	Not sequenced[a]	Total
L. cuprina	75	23	4	3	19	124
L. sericata	26	33	1	0	13	73
Total	101	56	5	3	32	197

[a]28S rRNA region of the nuclear genome could not be successfully sequenced.

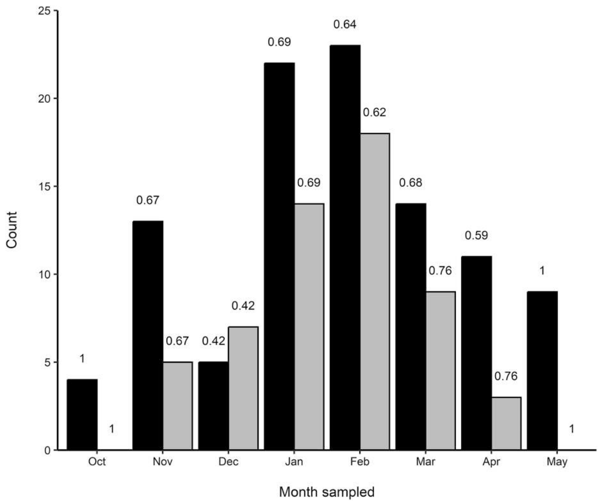

Figure 2. The count, by month of collection of *Lucilia cuprina* (black bars) and *Lucilia sericata* (grey bars) flies, identified by sequencing of the 28S rRNA gene, on eight farms in New Zealand in a study assessing the accuracy of identification of these species. Above each column, the accuracy of morphological identification for the respective species and month is shown. There were no *L. sericata* flies collected in October and May.

a comparison of the humeral callus and the surface of the notopleuron for *L. cuprina* and *L. sericata*. The images are from flies identified molecularly.

Twenty of the longest reads (17 *L. cuprina* and 3 *L. sericata* sequences) were submitted to the National Centre for Biotechnology Information (accession numbers PP869213–PP869232).

Discussion

The key aim of this study was to assess morphological and molecular methodologies that may be used to distinguish *L. cuprina* and *L. sericata*. It was found that the accuracy of the morphological identification in this study was about 70% for these species when compared to the results from sequencing the 28S rRNA region. These results show that caution should be used when relying solely on morphological identification, especially when there are large collections of Diptera stored in ethanol to identify, which may also be damaged or decomposed, and it would be advisable to regularly use molecular methods to monitor identification accuracy.

There are several reasons for the potential limitation of only using morphology to identify these species. For example, the colour of the fore femora depends on the maturity of flies at collection, and whether they are damaged, and the relative width of the frontal stripe is more reliable in males than females (Waterhouse and Paramonov 1950). In addition, the number of occipital hairs and the fine hairs on the humeral callus cannot be accurately counted unless specimens stored this way are air-dried, and the metasternal area is exceedingly difficult to view if the legs are not positioned correctly (Williams and Villet 2014). Therefore, a suite of characteristics should always be employed for identification of *Lucilia* species (Akbarzadeh *et al*. 2015; Lutz *et al*. 2018; A Heath[2], pers. comm.). However, despite adopting this approach, misidentifications of *L. cuprina* and *L. sericata* still occurred in the present study. Importantly, the re-examination of a sample of flies after molecular identification using enhanced magnification and photography found that the morphological keys were accurate. The enhanced magnification and photography allowed for a more thorough examination of the specimens and helped overcome some of the negative effects of ethanol storage. However, this method would have been impractical and too slow for identifying all the many flies collected in the study.

Since the traps were only emptied once a week, it is possible that fly damage or post-mortem changes

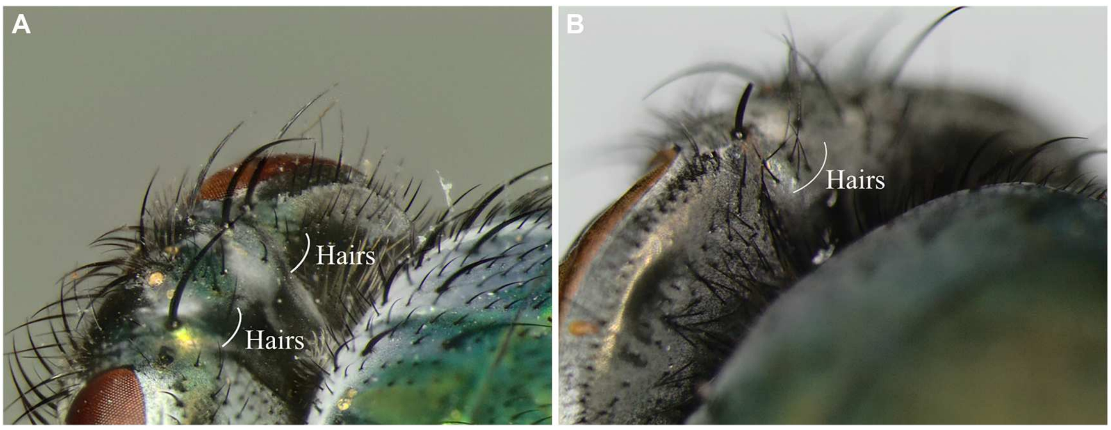

Figure 3. Photograph of a posterior view of the central occipital area of the head of flies identified by sequencing of the 28S rRNA gene as *Lucilia cuprina* (A) and *Lucilia sericata* (B) showing that *L. sericata* has more occipital hairs than *L. cuprina*.

[2]A Heath; AgResearch Ltd, NZ.

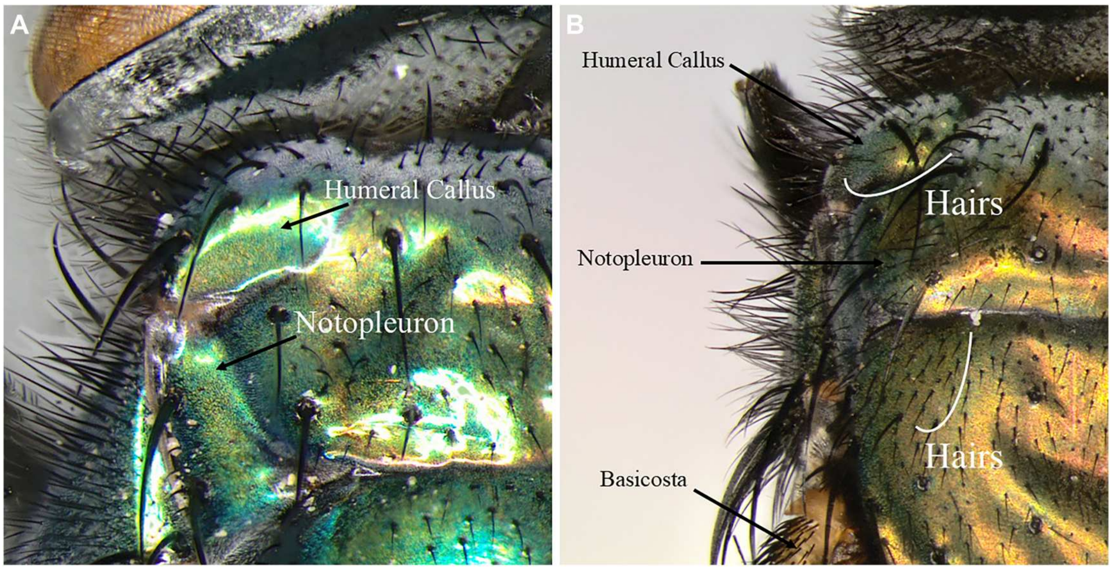

Figure 4. Photograph of a posterior view of the thorax of flies identified by sequencing of the 28S rRNA gene as *L. cuprina* (A) and *L. sericata* (B) with the humeral callus and notopleuron indicated showing that *L. sericata* has more hairs on the humeral callus and edge of the notopleuron than *L. cuprina*.

occurred, again making morphological identification more challenging. However, the study tried to overcome this by selecting only well-preserved specimens, but since the specimens were initially stored in ethanol at room temperature, those irreversible changes associated with ethanol storage would still have been present. Difficulty in morphologically distinguishing other *Lucilia* species, such as *L. caesar* and *L. illustris* has previously been noted (Velasquez *et al*. 2010; Szpila 2012).

The decision to molecularly confirm the morphological identification was made after the field surveys were completed and the flies were already stored in ethanol. In hindsight, storing the Diptera in dry conditions or freezing them would have been more appropriate, since this would have safeguarded their quality and preserved the integrity of the DNA. However, it is acknowledged that there can be issues with freezing: the thawed flies are often wet, and appendages can easily break off when frozen.

The results confirmed that there is a difference in the seasonality of these two fly species in New Zealand. It seems that *L. cuprina* emerges about 1 month earlier and persists for approximately 1 month later than *L. sericata* at the end of the season (see Figure 2). This finding is consistent with earlier reports that were based on the collection of larvae from live fly-blown sheep (Heath and Bishop 1995, 2006). This means that the flystrike season in New Zealand has likely extended by approximately 2 months since the arrival of *L. cuprina* in the late 1970s.

These results support the use of complementary molecular methods to improve the accuracy of morphological identification, especially when the specimens are stored in ethanol. While Sanger sequencing of PCR amplicons is the gold standard method of fly identification, it is too time-consuming and expensive for routine use. Therefore, an alternative cost-efficient molecular method which can rapidly identify *L. cuprina* and *L. sericata* needs to be developed. This is especially important where field specimens are damaged, decomposed or poorly stored, and need to be accurately identified. Suitable potential molecular techniques include quantitative PCR and loop-mediated isothermal amplification (LAMP). These techniques have the advantage of being completed within an hour and are commonly used to detect mosquitos in a cost-effective manner (Thabet *et al*. 2022) or as part of wider vector surveillance programs for mosquito-vectored diseases (Fynmore *et al*. 2021; Dieme *et al*. 2022).

Conclusion

Using morphological methods alone can be a self-fulfilling prophecy, since there is no reference test to challenge the veracity of the results and, although our storage methods may have negatively impacted its accuracy, this study has potentially identified limitations in correctly identifying *L. cuprina* and *L. sericata* using morphology. Inaccurate identification of flies could have negative repercussions for the prevention and control of flystrike in sheep across New Zealand, given that global warming is likely to extend the flystrike season. The results also showed that *L. cuprina* already has a longer season than *L. sericata* in New Zealand by up to 2 months.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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