Insect Conservation and Diversity (2013) doi: 10.1111/icad.12024

Impact of non-lethal genetic sampling on the survival, longevity and behaviour of the Hermes copper (*Lycaena hermes*) butterfly

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Abstract. 1. Genetic techniques are important tools for conservation, but tissue sampling for DNA analysis can be particularly detrimental to small study organisms. Historically, obtaining DNA samples from small insects and butterflies has involved destructive (lethal) methods.

2. Recent improvements to DNA purification technologies have increased the likelihood that non-lethal sampling will be successful. In spite of this, only a few studies have evaluated the impacts of sampling on survival and behaviour.

3. The Hermes copper, *Lycaena hermes* (Edwards), butterfly has a restricted distribution and generally less than 10 individuals are encountered at any one location. Non-lethal DNA sampling would allow for genetic studies that have the potential to augment conservation decisions without causing local extirpations.

4. We demonstrate that removing a leg from an adult male Hermes copper does not have a measureable effect on their survival, longevity or behaviour. In addition, a single leg provides a sufficient DNA sample for amplified fragment length polymorphism studies.

5. The Hermes copper butterfly represents the smallest butterfly species for which the survival and behaviour has been assessed in relation to non-lethal tissue sampling. This suggests that research involving smaller and more delicate species could utilise leg removal as a non-lethal genetic sampling technique.

Key words. Amplified fragment length polymorphism, conservation genetics, Hermes copper (*Lycaena hermes*) butterfly, mark-release-recapture, movement, non-lethal sampling, survival.

Introduction

A number of recent studies have explored non-lethal genetic sampling for insects and generally involved wing-clips and leg removal (Rose *et al.*, 1994; Keyghobadi *et al.*, 1999, 2006, 2009; Fincke & Hadrys, 2001; Holehouse *et al.*, 2003; Châline *et al.*, 2004; Hadrys *et al.*, 2005). Despite the increasing use of these non-lethal sampling techniques, rarely is the impact of the tissue sampling on survival or behaviour assessed. The few studies addressing impacts on these parameters demonstrate that wing-clips and leg removal have no noticeable effects on survival and/or behaviour, especially in butterflies (Roland *et al.*, 2000; Hamm *et al.*, 2010; Koscinski *et al.*, 2011). Despite this, exceptions exist with other insect groups. Starks and Peters (2002) found increased mortality when removing the distal portion of a wasp's leg and Vila *et al.* (2009) found that female moths had lower mating success after leg removal.

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As genetic studies can greatly assist management and conservation efforts it is integral to develop non-lethal sampling techniques. For large organisms, like most vertebrates, genetic samples can be obtained without killing the organism. But, for small bodied and/or delicate insects with small population sizes it is imperative to develop non-lethal techniques that will not contradict fundamental conservation ideals.

The Hermes copper, *Lycaena hermes* (Edwards), is a small butterfly with a wingspan of 2.5–3.2 cm (Opler *et al*., 2011). The Hermes copper butterfly exhibits a restricted distribution in the coastal sage scrub habitat of San Diego County, CA USA and northern Mexico (Marschalek & Klein, 2010). This species is rarely encountered within the limits of this distribution, resulting in concern about the status of the species (Marschalek & Klein, 2010; U.S. Fish Wildlife Service, 2011). In nearly all cases, surveys conducted for the duration of each flight season in 2008–2012 have yielded fewer than 10 Hermes copper butterflies within local populations (unpublished data by DM). Non-lethal genetic sampling is important for genetic studies, which have the potential to assist conservation efforts, without that same research causing local extirpations.

Due to the small size of the Hermes copper butterfly, it is reasonable to hypothesise that genetic sampling involving this species could result in a higher risk of mortality during sampling compared to larger species. For this reason it is important to confirm that sampling techniques which are non-lethal for other butterfly species do not negatively impact the survival or behaviour of the Hermes copper. In addition, it is important to confirm that any sampled tissue will provide sufficient DNA to conduct genetic studies. We assessed the impact of leg removal on Hermes copper survival, longevity and behaviour. We also report the suitability of a non-lethally obtained genetic sample for use in the amplified fragment length polymorphism (AFLP; Vos *et al*., 1995) technique. Our findings will be applicable to other butterfly species of similar size and delicate structure.

Methods

We conducted a mark-release-recapture study at Sycuan Peak Ecological Reserve in San Diego County, CA USA to assess the impact of presumed non-lethal sampling (leg removal) on adult survival, longevity and behaviour. Surveys were conducted every other or every third day for the duration of the 2011 adult flight season (31 May–1 July). The Hermes copper poses two biological limitations for this type of research: (i) the species is extremely rare, and (ii) females are rarely observed, likely due to secretive behaviour. To address the rarity of this species, we chose this study site because it is the largest known population of Hermes copper butterflies (unpublished data by DM). We excluded females from this study due to few sightings coupled with low resighting rates of females, which is consistent with past marking studies (Marschalek & Deutschman, 2008; Marschalek & Klein, 2010). Excluding females also provided the benefit of limiting negative impacts on this local population.

We uniquely marked all captured Hermes copper males with a felt-tipped marker (Ehrlich & Davidson, 1960) and recorded locations of all sightings with a handheld Garmin 76™ GPS unit. This GPS has an accuracy of within 3 m as determined by the manufacturer, but our work has demonstrated an error of <0.5 m. One prothoracic leg was removed (as a genetic sample) from approximately every other male Hermes copper butterfly captured during each survey. Survival was assessed by resighting rates and longevity was assessed by the minimum number of days an individual was known to be alive. Only resighted males were included in the longevity analyses. We inferred impacts on behaviour using movement data as well as opportunistic observations.

We used χ^2 tests to detect differences in resighting rates and a two-sample *t*-test to detect differences in longevity and movement data. Because longevity and movement data are right-skewed, we corrected these data with a log +1 transformation to achieve normality. Reported values involved a back transformation to a non-logarithmic scale.

To evaluate if a single Hermes copper butterfly leg provides a sufficient quantity of DNA for AFLP studies, DNA was extracted and purified using a DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). Afterwards, we used a sodium acetate/ethanol precipitation to concentrate the DNA sample. We measured DNA concentration using UV absorbance spectroscopy. Following AFLP procedures in Berres (2003), we assessed the replicability of AFLP markers with the primer combination EcoRI + GT/AseI + TG. Our trials show that this primer pair yields a large number of AFLP markers that are well distributed in the 50–620 base-pair length for the Hermes copper. Quality assurance tests involved DNA extracted from whole-bodies so that leg samples could be compared to AFLP fingerprints generated from the same individual. This test involved individuals collected in 2010. We used the trace analysis program DAx 8.0 (Van Mierlo Software Consultancy; Eindhoven, the Netherlands) to visualise AFLP fingerprints and to identify and compute the molecular mobility of individual markers.

Results

We marked a total of 58 adult male Hermes copper butterflies. The prothoracic legs (forelegs) were more easily pulled off compared to any other legs. For this reason we made efforts to remove either prothoracic leg in an attempt to reduce further potential negative impacts to individual butterflies. We found no significant difference in resighting rates ($\chi^2_1 = 0.62$, $P = 0.430$) or minimum number of days known alive (control = 6.43 day, 4.75–8.62 95% CI, $n = 14$; leg removed = 5.65 day, 4.20–7.51

95% CI, $n = 15$; $t_{27} = -0.669$, $P = 0.509$) between the two groups (Table 1). The longest recorded lifespan was 15 days and included individuals with and without a leg removed. Our measurement of longevity likely underestimates the actual length because individuals may escape detection by being alive on days not surveyed or leaving the survey area.

No significant difference in movement was found for either distance between consecutive sightings (control = 10.53 m, 6.00–18.01 m 95% CI, $n = 34$; leg removed = 13.93 m, 6.40–29.20 m 95% CI, $n = 24$; $t_{56} = 0.635$, $P = 0.528$) or total distance travelled (control = 35.22 m, 13.66–88.72 m 95% CI, $n = 14$; leg removed = 29.06 m, 10.89–75.03 m 95% CI, $n = 15$; $t_{27} = -0.309$, $P = 0.760$) (Table 1). Field observations also provided evidence that removing a leg has minimal impacts. Resighted males with a leg removed were found defending territories for several days. Also, copulation was observed less than 2 hour after marking and removing a leg from a specific male, suggesting that males can still mate successfully without a prothoracic leg.

DNA isolated from legs yielded an average of 1273.3 ng ($n = 27$, range = 180–2940 ng) of DNA. This is nearly three times greater than the 500 ng used as starting material for the original development of the AFLP technique (Vos *et al.*, 1995). In addition, our trials indicate that AFLPs can be successful and reproducible starting with 50–100 ng of DNA. We were able to generate reproducible AFLP fingerprints with DNA purified from all 27 leg samples. For the quality assurance test, AFLP fingerprints generated from a leg were identical to those traces generated from the whole-body of the corresponding individual.

Discussion

We demonstrated that removing a prothoracic leg from Hermes copper butterflies had no measurable impact on their survival, longevity and behaviour. This is consistent with the other studies investigating non-lethal genetic sampling for various insect species (Keyghobadi *et al.*, 2009; Hamm *et al.*, 2010; Koscinski *et al.*, 2011). Importantly, Hermes copper represents the smallest butterfly species for which the impact of non-lethal genetic sampling on survival and/or behaviour has been assessed thus far (Koscinski *et al.*, 2011).

Although handling of butterflies is a concern (Singer & Wedlake, 1981; Morton, 1982), high resighting rates with the Hermes copper (Marschalek & Deutschman, 2008; Marschalek & Klein, 2010) suggest that any negative impacts from handling is not a problem in our case. It should be noted that the rarity of the Hermes copper butterfly and virtually no resightings of females provide statistical limitations related to sample sizes. This is likely reflected in the range of confidence interval values. Although we may not be able to detect small differences in the survival and mortality parameters, our data show that leg removal does not have a large impact. Even if leg removal does have a slight effect on survival and behaviour, it is likely negligible compared to the variability caused by environmental factors (e.g. rainfall, temperature).

Because lack of statistical power can result from small sample sizes, we conducted a power analysis (Lenth, 2006–2009) using an 80% confidence level to determine the effect size required for a statistical difference between the two groups. With our sample sizes, differences in at least 3.41 days, 16.35 and 65.33 m for minimum days alive, distance between consecutive movements and total distance moved, respectively, would be required for statistical significance ($\alpha = 0.05$). If leg removal does impact Hermes copper butterfly longevity or movement, it is less than the values estimated by our power analysis.

We chose to investigate leg removal and not wing-clips because we felt wing-clips would have increasingly negative effects as the size of the butterfly decreased. As wing size decreases, a given area clipped represents a larger proportion of the total wing area, resulting in increased wing loading. A higher wing load increases energetic requirements while in flight and may slow flying speed which could be important for a number of daily activities (e.g. predator avoidance, territory defence). Although not assessed in this study, wing-clips may still offer an option for non-lethal tissue sampling for smaller butterflies such as the Hermes copper. Other researchers have noted that wing-clips resemble normal wear or damage due to bird attacks (Lushai *et al.*, 2000). We did observe Hermes copper butterflies with slightly damaged wings and their flight appeared unaffected. If wing-clipping for smaller insects does become problematic, leg removal is a viable option.

An ability to obtain genetic samples that yield sufficiently large amounts of DNA from small insects without impacting their behaviour or survival is a valuable tool

Table 1. Survival and movement data of adult male Hermes copper butterflies, comparing those only marked (control) with those marked and one leg removed (leg removed).

	Individuals	Number resighted	Minimum days alive	Maximum lifespan	Distance between consecutive sightings	Total distance moved
Control	31	14 (45.2%)	Mean = 6.43 day SE = 5.59–7.38	15 day	Mean = 10.53 m SE = 8.02–13.76	Mean = 35.22 m SE = 22.82–54.08
Leg removed	27	15 (55.6%)	Mean = 5.65 day SE = 4.93–6.46	15 day	Mean = 13.93 m SE = 9.62–19.99	Mean = 29.06 m SE = 18.50–45.34

for conservation. In the case of the Hermes copper butterfly, adult numbers are very low even at the height of the flight season (Marschalek & Klein, 2010). Obtaining appropriate sample sizes with destructive sampling creates ethical dilemmas as the persistence of these small local populations could be jeopardised. The ability to increase sampling of small insects such as the Hermes copper provides enhanced opportunities for genetic studies, particularly those that require more DNA such as AFLP and next-generation sequencing. Genetic information, combined with other ecological and biological studies, can augment conservation and management decisions, and could have future use with breeding programmes (Châline *et al.*, 2004).

We recommend confirming replicability of any genetic technique when exploring the use of increasingly smaller tissue samples and particularly when using smaller amounts of DNA. A single Hermes copper leg provides sufficient DNA for many genetic techniques, including AFLP which tends to require more starting material than other techniques such as mtDNA sequencing and microsatellites. Applicability of the AFLP technique is particularly important as microsatellites can be problematic for use in Lepidoptera (Zhang, 2004). Moreover, AFLP allows a relatively quick and cheap method to characterise non-model species (Meudt & Clarke, 2007).

Despite the Hermes copper butterfly being relatively small and delicate, we have shown that it can tolerate the impacts of leg removal. But, we recommend assessing non-lethal sampling on other small insects first, prior to wide-scale implementation. Given the scarcity of reports such as this work, it remains possible that other species may react differently to leg removal. It is also important to consider the ability of the researcher to handle, sample and release the individual while minimising any harm to the individual insect.

Acknowledgements

We thank San Diego Association of Governments for supporting this research, and the California Department of Fish and Game for granting permission to conduct this study at Sycuan Peak Ecological Reserve. We also thank two anonymous reviewers who provided comments on the original manuscript.

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Accepted 7 January 2013

Editor: Simon R. Leather
Associate editor: Karsten Schonrogge