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Collecting blood samples in Eurasian Kestrels (*Falco tinnunculus*) (Aves: Falconidae) via blood-sucking bugs (Insecta: Hemiptera: Reduviidae) and their use in genetics and leucocyte profiles

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Abstract

We modified the non-invasive technique to bleed incubating birds via the blood-sucking bug *Dipetalogaster maximus* in an artificial egg for an urban breeding raptor, the Eurasian kestrel (*Falco tinnunculus*) in Vienna, Austria, in 2012. We evaluated the use of the technique for the purpose of: (1) identification of the breeding bird, (b) microsatellite analysis for paternity tests, (3) the count of blood parasites and (4) leucocyte profiles based on blood smears. We can conclude that the method is useful for identification of individuals, microsatellite analyses and blood parasite counts, as long as the bug-egg is retrieved after not later than 4 hours from the kestrel clutch and needs further evaluation for leucocyte profiles.

Key words: *Falco tinnunculus*, blood screening, artificial egg, blood-sucking bug, *Dipetalogaster maximus*, non-invasive bleeding.

Zusammenfassung

In dieser Arbeit wird eine nicht-invasive Methode zur Blutabnahme bei brütenden Vögeln über die Raubwanze *Dipetalogaster maximus* modifiziert. Dazu wurden 2012 in den zu untersuchenden Nestern der in Wien brütenden Turmfalken (*Falco tinnunculus*) künstliche Eier mit *D. maximus* Larven platziert. Diese Methode zur Blutabnahme ohne Störung des Brutgeschehens diente (1) der Identifizierung der Brutvögel, (2) der Mikrosatellitenanalyse für Vaterschaftstests, (3) dem Erfassen von Blutparasiten und (4) und der Erstellung eines Leukozytenprofils mittels Blutausstrichen. Zusammenfassend kann gesagt werden, dass diese Methode für die Identifizierung von Individuen, zur Mikrosatellitenanalyse und für das Auszählen von Blutparasiten gut geeignet ist, vorausgesetzt die Raubwanze ist nicht länger als 4 Stunden im Turmfalkengelege. Die Untersuchung von Leukozytenprofilen muss im Detail noch ausgetestet werden.

Introduction

For many endocrinological, physiological and genetic investigations blood samples are an indispensable source. However, during breeding season, obtaining blood samples from incubating birds is difficult and time consuming, and trapping in wild populations often restricted in endangered or protected species (Kania 1992). Even if trapping at the nest-site is feasible, it causes stress that may negatively affect breeding success or even cause nest desertion. It may also alter blood chemistry as levels of plasma corticosterone rise immediately following capture (Romero & Romero 2002, Romero & Reed

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2005). Consequently, there is a high interest in minimal or non-invasive techniques to bleed wild birds without catching and handling (Becker et al. 2006, Arnold et al. 2008, Bauch et al. 2010, Bauch et al. 2013b).

Helversen & Reyer (1984) described a method for obtaining blood from animals using Triatominae bugs, blood-sucking insects belonging to the family Reduviidae which includes *Dipetalogaster maximus* (Uhler, 1894). The efficacy of blood-sucking bugs has since been validated for doubly-labelled water experiments (Voigt et al. 2003), determination of progesterone, testosterone and hydrocortisone concentrations (Voigt et al. 2004), serological studies with virus-neutralising antibody titres (Voigt et al. 2006, Vos et al. 2010), determination of prolactin and corticosterone (Arnold et al. 2008, Riechert et al. 2012), measurement of a pregnancy hormone in the Iberian Lynx (*Lynx pardinus*) (Braun et al. 2009) and recently also for obtaining leucocyte profiles in the animal laboratory on Rabbits (*Oryctolagus cuniculus*) (Markvardsen et al. 2012) and to investigate telomere lengths in avian erythrocytes (Bauch et al. 2013a).

Becker et al. (2006) modified this technique for incubating Common Terns (*Sterna hirundo*) by using a blood-sucking bug in a hollow egg. It was also successfully applied to smaller bird species, like Common Swift (*Apus apus*) (Bauch et al. 2013b). These studies did not find differences in hormone levels obtained via various sampling methods, but the bug-method still needs validation in a field setting (Arnold et al. 2008). Becker et al. (2006) also speculated about a possible effect of the bug's intestinal liquids on the DNA of the focus animal. Overall, the method has the benefit of getting a blood sample without risking haematomas and damage to the blood vessel, which is extremely important in wild animals.

We used the "bug-egg" technique to study a wild urban Eurasian Kestrel population *Falco tinnunculus* in Vienna, Austria (48°12'N, 16°22'E). We tested whether this method is feasible for large-scale extraction of blood samples for (1) individual identification of the breeding bird, (b) microsatellite analysis for paternity tests, and (3) detection of parasites in blood taken from the bug's gut. Kestrels in our study area are non-colonial breeders and their nests are mostly situated in cavities and facades of buildings difficult to access. In that urban setting blood sampling cannot be done within the required three minutes of first disturbance (Romero & Reed 2005). Hence we also test (4) the use of the blood smears for obtaining leucocyte profiles (Heterophils/Lymphocytes-ratio) as an alternative to the concentration of circulating glucocorticoids as a measure of stress (Müller et al. 2011).

Material and methods

The artificial "bug-egg"

Fieldwork was carried out in 2012. For the construction of artificial Kestrel eggs, we followed the instructions of Becker et al. (2006). We produced small (35.0 × 29.8 mm) and large eggs (38.5 × 31.0 mm), based on measurements taken in the field in 2010–11 (n = 45), in species-specific colour patterns as evident from eggs in the Bird Collection in the Museum of Natural History in Vienna (Fig. 1). We used epoxy casting resin for the shell and polyurethane foam to make the egg lightest on the side where the bug can pierce the grid. This ensured contact with the incubation patch of the breeding bird also

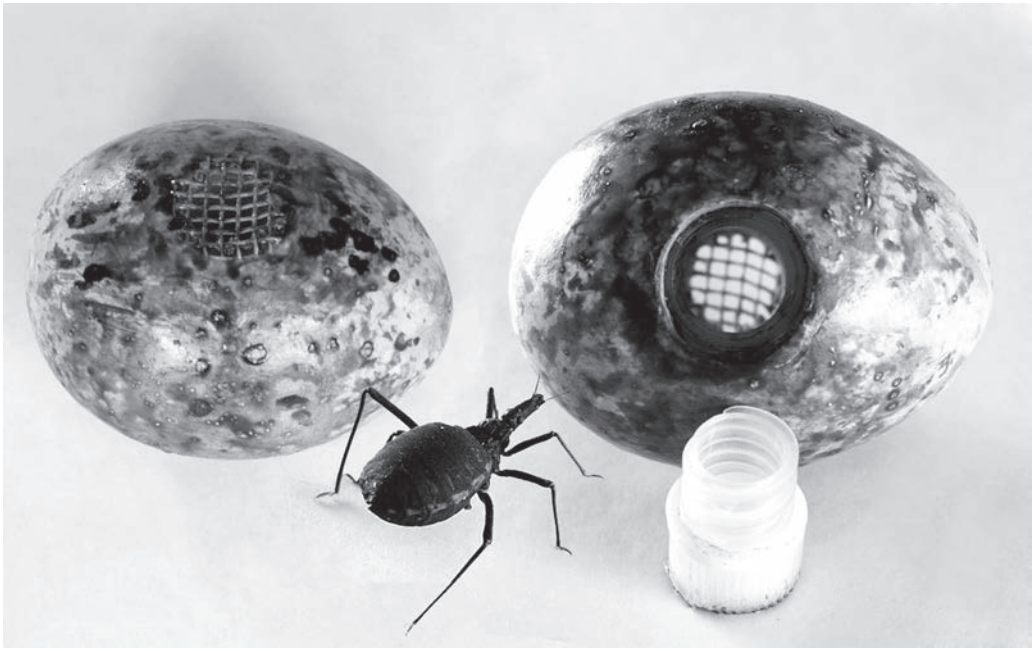

Fig.1: The artificial egg from top (smaller model, left) and bottom (larger model, right) and fully sucked larval instar L3 of *Dipetalogaster maximus* (photo: G. Witting).

during egg-role behaviour. We wanted to find another solution than additional small holes throughout the shell of the egg (used by ARNOLD et al. 2008 and BAUCH et al. 2013b) to ensure that no sand from the ground of the breeding cavity is blocking the holes and also to increase the stability of the artificial egg.

Since Kestrel nests in the city are hard to reach (mostly via facade and tree climbing) we did not replace the eggs by dummies during the procedure to avoid any damages to the clutch. We just added one artificial egg within the produced clutch, choosing the one closest in size and colour to the existing clutch (Fig. 2) for the same reasons as mentioned before. This was recently also successfully done by BAUCH et al. (2013b) in Swifts.

For the blood-sucking procedure we used starved third larval instars (L3) of *D. maximus* (ordered from AG Zoology/Parasitology, Ruhr-University-Bochum; contact person G.A. SCHAUB). We tested the bug's willingness to sting by presenting a finger at a few centimetres distance. A hungry bug immediately approached with the proboscis erected (see also BECKER et al. 2006). Until application, L3 were kept under laboratory conditions at 27°C and 70 % relative humidity. Previous work in Common Terns (BECKER et al. 2006, ARNOLD et al. 2008) showed that a full blood meal could be obtained within 10 min, but the difficult access to Kestrel nests in our study area only allowed a nest check every 3–4 hours. The trial was done at 26 nest-sites between 22 April and 17 June 2012. Bugs were used once.

After collecting the artificial egg, we punctured the bug's abdomen with a needle (Gauge 27, ½ inch) to prepare a blood smear; the remaining sample (100–200µl) was stored at

Fig. 2: An artificial egg prepared with a blood-sucking bug placed in the middle of a complete Kestrel clutch (6 eggs) in a building cavity in Vienna, Austria (photo: P. Sumasgutner).

-20°C. We also sampled 154 Kestrel chicks by puncturing the brachial vein ("conventional method"). Although the bugs add the protein dipetalogastin as an anticoagulant (Lange et al. 1999), we stored all samples in EDTA-coated tubes to be able to use the same DNA extraction protocol.

Genetic analysis – sexing and paternity tests

Genetic analysis was conducted at the Laboratory of Molecular Systematics of the Museum of Natural History Vienna. We used the QIAGEN DNeasy Blood & Tissue Kit following standard protocol with Proteinase K to extract DNA. To confirm that we got blood from the incubating female we used the 2718R and 2550F primer set (Fridolfsson & Ellegren 1999) on 2% Agarose Gel. For paternity tests we used 14 different microsatellites established for *Falco peregrines* (Nesje et al. 2000) and *Falco naumanni* (Padilla et al. 2009): NVH fp5, NVH fp79–4, Fnd1.1, Fnd1.2, Fnd1.3, Fnd1.5, Fnd1.6, Fnd1.7, Fnd1.8, Fnd2.1, Fnd2.2, Fnd2.3, Fnd2.4, Fnd2.5. PCR reactions were performed using the QIAGEN Multiplex PCR Kit with 20–60 ng DNA following standard protocol. PCR cycles consisted of an initial activation step at 95°C for 15 min (HotStartTaq DNA Polymerase) and 35 cycles of 94°C for 30 sec, 48°C–57°C annealing temperature for 90 s and 72°C for 90 s, followed by a final extension of 72°C for 20 min. Differences in

final allele sizes and in fluorescent dye labels of primers allowed for pooling multiple loci. The pooled products were then diluted with water 1:20, mixed with HiDiFormamid (Applied Biosystems) and internal size standard LIZ500 and run on an ABI 3130×1 sequencer. All loci were visually identified using the program ABI Peak Scanner 1.0. Final allele sizes were determined using the binning software TANDEM 1.01 (MATSCHINER & SALZBURGER 2009). CERVUS 3.0 (KALINOWSKI et al. 2007) was used to determine expected (H_E) and observed (H_O) heterozygosities, and the overall probability of identity (PID). MICRO-CHECKER 2.2.3 (VAN OOSTERHOUT et al. 2004) was used to test for the possibility of scoring errors, allelic dropout and null alleles. We included all breeding females and one chick per nest-site (with the purpose to exclude full siblings) in these tests to reduce sampling effects due to relatedness among birds.

Blood-smears – blood-parasites and leucocyte profiles

Immediately after puncturing the bugs' abdomen or the hatchlings' brachial vein a small drop of blood (~10 µl) from the capillary tube was smeared and air-dried on a glass slide directly in the field to prepare a thin blood film. The blood smears were subsequently fixed with absolute methanol and stained with Giemsa's stain (using Hemacolor® Rapid staining of blood smear set). The coloured blood smears were screened by inspecting them under a light microscope at 400× magnification for at least 10 min to see if any blood parasites were visible. Then haematozoa were quantified by counting the number of parasites within 10 000 erythrocytes at 1000× magnification under immersion oil (50–100 fields). The fields were chosen in a line from one end of the slide to the other to compensate for differences in blood thickness across smears. Additionally a complete blood count was done to obtain information about the kinds and numbers of cells in the blood, especially erythrocytes, leucocytes and thrombocytes.

The sampling of nestlings was done at an age of 2–3 weeks. To get relative proportions of each leucocyte type, screening of the blood smear was continued until 100 leucocytes were examined (at 1000× magnification). Within these 100 leucocytes the actual number of heterophils (het), lymphocytes (lymphs), basophils (baso), eosinophiles (eos) and monocytes (mono) were counted. To separate this analysis from the count of haematozoa and the complete blood count we use the term leucocyte profile.

Results and discussion

Blood samples via blood-sucking bugs

Twenty-six nests were tested with bugs of which starvation time before the trial varied between 2–8 weeks, resulting in 21 samples from breeding females. For bugs with a starvation time of 2–4 weeks, success rate of blood-sucking in eight nests was 1 out 8 after 3–4 h, with another three after 6-8 h and three more after 10–12 h (replacing the bug after each nest check). After 6-8 weeks of bug starvation, success rate was markedly higher with 14 out of 18 having sucked blood within 3–4 h of exposure (summary on success rate in Table 1). STADLER et al. (2011) provided a detailed description how to raise and keep blood-sucking bugs, including the instruction that L3 can be used from 3–6 weeks after hatching onwards. In our trial we needed to extend that starvation time up to 6–8 weeks to successfully collect blood samples.

We found no visible response of the bird to the bug bite, but only two nest-sites could be directly observed during the whole trial period. In one case (3.4%) out of 26 trials the incubating bird disposed the artificial egg by removing it from the nest cup and destroying it. In general animals exposed to *D. maximus* do not seem to react to the bite, probably due to the small diameter of the proboscis (20 µm, smaller than a 26 Gauge needle) or to anaesthetic saliva being injected into the wound (Markvardsen et al. 2012). The use of this method had no negative effect on breeding success, as all chicks hatched successfully.

Sexing and paternity tests

In Eurasian Kestrels the sequence on the Z chromosome measures 484 base pairs (bp) and on the W chromosome 295 bp (Nittinger 2004). The difference of 189 bp was sufficiently long to detect both fragments on a 2% Agarose gel, without any visible relicts of DNA of *D. maximus*. Since the female is the heterogametic sex in birds, results show two bands for female blood, one band for male blood, but also two bands for a mixture of both. In kestrels risk of such a contamination remains low, since it is mostly the female incubating (Village 1990). A contamination would anyhow become visible on peak scan in our further analyses, which was apparently the case in 3 out of 21 samples we consequently excluded.

Four loci displayed significant deviations from Hardy–Weinberg equilibrium (P_{HWE}) or significant probability of null alleles (P_{null}): NVH fp79-4 ($P_{HWE} = 0.0311$, $P_{null} = 0.1435$), Fnd1.2 ($P_{HWE} < 0.0001$, $P_{null} = 0.0999$), Fnd1.6 ($P_{HWE} < 0.0001$, $P_{null} = 0.0505$) and Fnd1.8 ($P_{HWE} = 0.0011$, $P_{null} = 0.1902$). These loci were not used in further analysis. Our Kestrels

Table 1: Success rate of the blood-sucking bug *Dipetalogaster maximus* in collecting blood samples in Eurasian Kestrels (*Falco tinnunculus*) in Vienna, Austria in 2012, dependent of starvation and exposure time of the bug compared to cited bird studies using the bug-method: (+) successful, (-) not successful.

Species	**Success rate dependent on starvation and exposure time**			
Falco tinnunculus	Exposure time: Success rate:	3–4 h + / -	6–8 h + / -	10–12 h + / -
	2–4 weeks starvation	n = 1 / 7	n = 3 / 4	n = 3 / 1
	(n = 8 nests)	12.5%	42.9%	75%
	6–8 weeks starvation	n = 14 / 4	- / -	- / -
	(n = 18 nests)	77.8%		

Species	**Exposure time**	**Success rate**	**Starvation**	**Study**
Sterna hirundo	60 min	34% (n = 163)	no details	Becker et al. 2006
Sterna hirundo	30 min	89.5% (n = 14)	no details	C. Bauch unpubl. (in Arnold et al. 2008)
Sterna hirundo	30 min	86.1 (n = 34) 82.2% (n = 44)	no details	Bauch et al. 2010
Apus apus	60 min	40% (n = 27)	no details	Bauch et al. 2013b

showed polymorphism at all 10 tested loci, with the number of alleles per locus ranging from 8 to 16 and observed heterozygosities ranging from 0.570 to 0.882. With the remaining 10 microsatellites the females proved to be the genetic mother in all sampled nests, as also determined with the excluded loci Fnd1.2, Fnd1.6 and Fnd1.8, but not with locus NVH fp79–4. The "bug-method" can be used for the purpose of paternity tests, since we found no sign of the bug's DNA influencing the results.

Blood-parasites

We prepared 21 blood smears with the bug-method from breeding females and 154 with the conventional method from chicks. The screening of the blood-smears showed different qualities depending on the duration the bug-egg was left inside the nest: blood smears prepared after 3–4 h bug-egg's exposure in the nest were high in quality for blood-parasite counts. We only included these 15 samples in further analysis, since samples prepared after more than 6 hours contained a high quantity of lyced blood cells making it impossible to count infected red blood cells and to differentiate between different kinds of white blood cells to prepare a viable leucocyte profile (Table 2). VOIGT et al. (2004) experimented on digestion by bugs, showing an increased variation after 4 h of the blood meal. Even though the authors recommended using the sampled blood before 8 h after sampling for analysis, we needed to shorten that time in our study.

We detected *Haemoproteus* sp. in 46.7 % of adult breeding females (range of infected red blood cells within 10 000 erythrocytes: 13.7–34.1, median 21.0). For two *Haemoproteus* positive females we had samples collected via the bug method and the conventional method, revealing the same results: 16.0 vs. 20.7 parasites found in 10 000 erythrocytes on the smear and 34.0 vs. 34.1. Unfortunately, parasite prevalence in blood slides of

Table 2: Mean values (± SD) for complete blood counts within 10 000 erythrocytes and leucocyte profiles (Heterophils/Lymphocytes-ratio) within 100 leucocytes for breeding female Kestrels (*Falco tinnunculus*) in Vienna, Austria in 2012.

	Blood samples obtained via the bug-method		**t-value**	**P-value**	**Significance**
	Females (*n* = 8) *Haemoproteus* (-)	Females (*n* = 7) *Haemoproteus* (+)			
within 10 000 erythrocytes					
Leucocytes	65.3 (± 31.8)	119.7 (±103.4)	-4.48	0.0005	***
Thromocytes	2.6 (± 4.8)	15.1 (± 10.1)	-3.52	0.0034	**
within 100 leucocytes					
het	20.7 (± 12.8)	8.7 (± 8.3)	-4.57	0.0004	***
lymphs	73.3 (± 18.2)	86.6 (± 11.7)	-16.24	<0.0001	***
baso	0.6 (± 0.8)	2.2 (± 4.4)	-1.56	0.142	NS
eos	4.8 (± 4.9)	1.8 (± 3.0)	2.67	0.0182	*
mono	0.6 (± 1.1)	0.7 (± 1.3)	-0.49	0.6325	NS
H/L ratio	0.3	0.1	1.05	0.3103	NS

Significance codes: '***' 0.001, '**' 0.01, '*' 0.05, 'NS' not significant.

nestlings was nil, as we expected, because it takes at least 1–2 weeks for the parasites to be visible in the blood stream after infection of the nestling (Korpimäki et al. 1995). We presume that chicks older than two weeks might be infected with blood parasites but at very low prevalence. Parasites may not yet be fully developed in the blood cells, but their DNA might be detectable using PCR based methods instead of smears (Tomé et al. 2005, Delgado-V & French 2012).

Leucocyte profiles

To our knowledge there is only one study on haematology in blood sampled either via blood-sucking bugs or the conventional method done in laboratory with Rabbits (Markvardsen et al. 2012) and none in wild animals where the exposure time is harder to be standardised. Results therein have shown significantly higher levels of neutrophils, lymphocytes, basophils and monocytes in blood samples collected by the conventional method, concluding that a mixture of both methods is not accurate for H/L ratios. This difference might relate to (1) the metabolism of the bug, and/or (2) the bug blood samples originating from either a vein or an artery (see also Lue et al. 2007; conventional blood samples are collected from the veins). The study further concluded the exposure of the animal to bug antigens as unlikely to induce an immune response, since measured levels in leucocyte profiles should then have been higher in bug blood samples rather than the other way around. Since we only have both sampling methods for two individuals (results showed the exactly same H/L ratio for bug- and conventional-samples with a ratio of 0.02 for one and of 0.05 for the other female) we present results revealed via the bug method in incubating females. The H/L-ratio between infected (+) and *Haemoproteus* free (-) blood was not significantly different, but a logistic regression with the actual number of parasites in 10 000 erythrocytes and the H/L ratio showed a significant correlation: $R^2 = 0.29$, $F(1,13) = 5.28$, $P = 0.039$ of a lower H/L ratio in higher infected females. We further received significant values for less heterophils and eosinophils and more lymphocytes in *Haemoproteus* (+) individuals (Table 2) which are the basic measures resulting in the H/L ratio.

Conclusion

Our experiences have shown that the adaptations for the artificial eggs had clearly positive effects for the kestrel project. Using two materials with different density insured that the artificial eggs had a defined centre of mass and the opening with the L3 was always facing upwards, even during egg-roll behaviour. This method proved to be superior to fixing the artificial egg on the nest ground because it did not hinder the egg-roll behaviour of the female Kestrel and provides an alternative to holes all around the egg shell which might be problematic in a sandy breeding niche or when a higher stability is required. We can conclude that the stinging success of the L3s after 6-8 weeks of starvation was very high with 77.8 %. A shorter starvation time failed in our trial, which was previously only mentioned by Markvardsen et al. (2012) who used bugs fasted for 5 weeks prior to the study and failing to sting in their experiment. Further, the method is very useful for the purposes of microsatellite analyses and blood parasite counts, as long as the artificial bug-egg is retrieved not later than 4 h from the Kestrel clutch. For H/L ratios we clearly need further evaluations for differences in results obtained via the bug-method and the conventional method. In respect to applications necessary for field work,

our modifications proved unnecessary to replace all eggs with dummy eggs during the experiment making the procedure reasonably time-saving and safer in terms of preventing Kestrel eggs to be damaged or cooling during facade and tree climbing.

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