The applicability of spectrophotometry for the assessment of blood meal volume in artificially fed *Culicoides imicola* in South Africa

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ABSTRACT: The volume of the blood meal of hematophagous insects will determine the number of infective particles taken up during feeding and may as such denote the minimum dose needed to infect a competent vector. *Culicoides* midges resort amongst the smallest of hematophagous vectors and determining and comparing their blood meal volumes may be challenging. Collected *Culicoides imicola* females were fed on defibrinated bovine blood through a Parafilm® membrane using a Hemotek® system. After feeding, the weight of pools of 10 engorged females was compared to that of 10 unfed females to determine the volume of blood imbibed. After weighing, the pools were homogenised and their absorbance read at 410 nm. Spectrophotometer readings were then converted to blood meal volumes using calibration curves, obtained by the dilution of known volumes of blood used for feeding. Although the mean blood meal volumes determined spectrophotometrically (0.06 µl), differed significantly ($P < 0.01$) from those obtained by weighing (0.07 µl), the range in blood meal volumes determined spectrophotometrically (0.03 µl to 0.08 µl), and by weighing (0.01 µl to 0.11 µl) was positively correlated ($r = 0.7$; $P < 0.01$). Both methods can be used to determine the blood meal volume.

Keywords: Hematophagous insects, Hemotek® feeding system, livestock.

INTRODUCTION

Globally in excess of 75 viruses of medical and veterinary importance have been isolated from one or more of 1368 described species (Borkent, 2017) of blood feeding midges in the genus *Culicoides* Latreille (Diptera: Ceratopogonidae) (Meiswinkel *et al*., 2004; Purse *et al*., 2015). Based on a high co-abundance with livestock, host preference and confirmed oral susceptibility, the mammophilic *Culicoides* (*Avaritia*) *imicola* Kieffer is considered the principal vectors of bluetongue-, African horse sickness-, epizootic hemorrhagic disease- and

equine encephalosis viruses in South Africa (Meiswinkel *et al*., 2004). *Culicoides imicola* are furthermore one of the most widely distributed livestock associated *Culicoides* species in the world (Meiswinkel *et al*., 2004; Purse *et al*., 2015).

In the absence of laboratory colonies, the artificial feeding of field collected midges will be unavoidable in determining and comparing the oral susceptibility of various species and populations of *Culicoides*. Several artificial feeding methods, ranging from cotton wool pledgets soaked in virus infected blood, membrane feeding systems and the feeding of midges on infected embryonated chicken eggs were developed for the artificial feeding of *Culicoides* on virus impregnated matrices (Jones & Foster, 1966; Boorman *et al*., 1975; Van der Saag *et al*., 2015; 2017). It was, however, shown that oral susceptibility results can be influenced by the feeding method applied (Venter *et al*., 2005; Van der Saag, *et al*., 2015; Federici *et al.*, 2019). The volume of blood an insect consumes during feeding will determine the number of infective particles ingested and as such denote the minimum level of viremia needed in a host to infect a competent vector (Venter *et al*., 2005).

A frequently used method to determine the quantity of blood ingested consists of weighing insects before and after feeding. However, when using this method delays in reweighing after feeding may cause an underestimation of the volume of the blood meal due to the elimination of excess liquid during and after feeding (Leprince *et al.*, 1989). Previous studies in South Africa, based on weighing engorged and unfed females, calculated that the blood meal volume of *C. imicola* females fed via one-day-old chicken-skin membranes ranged from 0.023 µl to 0.062 µl with a mean of 0.045 µl (Venter *et al*., 2005). Using cotton wool pledgets, the blood meal volume ranged from 0.019 µl to 0.038 µl, with a mean of 0.038 µl (Venter *et al*., 2005). Taking into account that a number of factors can influence the feeding rate and volume of blood taken up during feeding (Lehane, 2005; De Beer *et al*., 2018), it will be essential to have a reliable and reproducible method to determine the volume

of blood taken up during artificial feeding in order to compare the vector competence of field collected species for various viruses (Wilson & Harrup, 2018). The applicability of spectrophotometry for this purpose was compared to the conventional weighing method.

Material and methods

Midge collection: Midges were collected overnight at the ARC-Onderstepoort Veterinary Research (ARC-OVR) (-25.6501, 28.1870; 1219 m above sea level) in South Africa in summer from February to April 2018 using down-draught, 220 V OVI light traps equipped with 8 W UV-light tubes placed near cattle as described by Venter *et al*. (1998). In the morning, beakers containing *Culicoides* were transferred into a black box with a ventilated lid and allowed to escape through a white translucent funnel into a 300 ml unwaxed paper cup (Venter *et al*., 1998). *Culicoides imicola* were identified using existing identification keys and species descriptions (Meiswinkel, 1989; Labuschagne, 2016). Collected midges were kept in the dark at 25 °C and fed a 10% (w/v) sucrose solution on cotton wool pledgets, which were removed 12 hours before subsequent blood feeding. The midges were kept in the dark to minimise the constant phototactic behaviour of the midges and to increase survival.

Midge feeding: A commercially available Hemotek® feeding system with a PS6 Power Unit equipped with a FU1 feeder loaded with 3 ml defibrinated bovine blood was used for feeding. No anticoagulants were added, and the blood was mechanically defibrinated on collection using of sterile 6 mm diameter glass beads. After defibrination the blood was stored in sterile 20 ml screw-top glass bottles at 4 ºC. Before feeding the top layer of plasma was removed with a 5 ml disposable syringe to prevent midges from feeding on plasma instead of the blood cells.

Immediately before feeding, midges were immobilized at -20 ºC for 30 seconds, pooled in groups of 1000 to 1500 individuals, and transferred to a custom-made feeding chamber. Pool sizes were approximated to eliminate sorting on a chill table before feeding, which in turn decreased handling and mortality. The feeding chamber consisted of a plastic container 45 mm in diameter and 74 mm high, with the bottom removed. The feeding chamber was connected securely to the FU1 feeder of the Hemotek® system before introduction of the immobilized midges, to prevent them from escaping. The connection of the feeding chamber to the feeder allowed direct contact between the midges and the stretched Parafilm® membrane, thereby eliminating the use of netting to secure the midges.

Contrary to the general procedures, used for routine mosquito feeding (Gunathilaka *et al*., 2017; Kauffman *et al*., 2017) and some bigger *Culicoides* species (Lilian *et al*., 2015), the FU1 feeder was used with the blood reservoir at the bottom of the feeding chamber instead of on top. The midges were acclimatised for at least 24 hours at 23.9 ±0.82 °C, 69.02 ±5.50 % RH in the dark and fed within 48 hours of collection. A feeding time of 35 minutes was allowed.

Blood meal volume assessment: Immediately after feeding, the *Culicoides* were immobilized at -20 ºC and randomly sorted into pools of 1, 10, 20 and 50 unfed and blood engorged *C. imicola* females, respectively, on a refrigerated chill table. The *C. imicola* were placed in an Eppendorf® and weighed. The blood meal weight was subsequently determined by subtracting the weight of a fixed number of unfed females from that of an equal number of engorged females. Twelve replicates with pools ranging from a single to 50 *C. imicola* were conducted. The weight was converted to volume using a conversion factor of 1.06 (Venter *et al*., 2005; Aqua-calc., 2020).

After weighing, the pools of engorged females were stored at -20 °C until homogenised using a TissueLyser (Qiagen®) in an Eppendorf® containing two stainless steel beads (3.0 mm diameter) and 120 µl distilled water. After homogenisation, the samples were vortexed and 100 µl of the sample transferred to an ELISA plate and the absorbance read with a SpectraMax spectrophotometer. The average of three readings per sample was used.

The absorption range at which the *Culicoides* should be read in the spectrophotometer was determined beforehand by using samples with a known blood concentration. The absorption rate peaked at a wavelength of 410 nm and all samples were subsequently read at this value. The mean spectrophotometer readings of the engorged midges were converted to blood meal volumes with the aid of calibration curves generated independently. These were generated by diluting 100, 90, 80, 70, 60, 50, 40, 30, 20, 10, 5, 3 and 1 µl of the blood that was to be used for feeding in a final volume of 100 µl, distilled water. The average of three readings per dilution was used to generate the calibration curve. To determine to what extent the absorption of the stored blood might change over time during storage, the blood dilutions were also evaluated seven and 38 days after collection.

After newly emerged female *Culicoides* have taken their first blood meal, and the subsequent production of the first egg batch, a burgundy pigment is deposited in the walls of the abdomen (Dyce, 1969). To assess the potential effect of this pigmentation on the absorption values obtained, pools of 10 unfed unpigmented (nulliparous) *C. imicola* females were compared with pools of 10 pigmented (parous) females. Ten replicates were conducted.

Statistical analyses: All data were analysed using the statistical software GraphPad Instat (version 3.00, 2003). For the conversion of spectrometry readings to blood meal volumes linear regression was used to construct calibration curves. For comparison of the blood meal volumes as determined by weighing and spectrophotometry a pairwise analysis of variance

(ANOVA) with a paired t test was used. Additionally, linear regression analysis was carried out on blood meal volumes obtained by weighing and spectrophotometry. All data was normally distributed, and statistical testing was conducted at the 5% significance level.

Results

Calibration Curves: Calibration curves were generated by a dilution range of known volumes of defibrinated bovine blood used for feeding to convert spectrophotometry absorption values to blood meal volume values. The slope of the calibration curve seven days after blood collection was 3.61 ($r^2 = 0.99$; $P > 0.01$; SD = 0.03). When the same blood was evaluated 38 days later, the slope decreased to 2.96 ($r^2 = 0.99$; $P > 0.01$; SD = 0.03). The significant decreases in the slope indicated that the absorption of the blood decreases over time and that a calibration curve needs to be generated on the day of feeding.

The influence of pool size on spectrophotometer readings: The influence of *C. imicola* pools size on the accuracy of the blood meal volume as determined by spectrophotometry absorption values was investigated. The absorption values obtained for pools of 1, 10, 20 and 50 *C. imicola* females were 0.001, 0.136, 0.513 and 0.761, respectively. Using a calibration curve, created from blood dilutions, these absorption values related to 0.01, 0.69, 2.62 and 3.85 µl of blood in these pools, respectively (Fig. 1). Based on these results and to increase the accuracy of the blood meal determination, a sample size between 10 and 20 *C. imicola* females per pool was used.

The influence of age structure on spectrophotometer readings: Despite a greater variation in unfed pigmented (parous) females, the average spectrophotometry absorption values obtained for unfed unpigmented (nulliparous) females (0.18 ± 0.11) did not differ significantly from

those of unfed pigmented females (0.27 ± 0.41) (Fig. 2). Both these values were significantly ($P < 0.01$) different from that of blood-fed females (1.06 ± 0.47) (Fig. 2). This indicated that the presence of pigment in the abdomen will not significantly increase the spectrophotometry absorption values.

Blood meal volume determination comparison: The volume of blood consumed by a *C. imicola* female as determined using weighing ranged from 0.01 µl to 0.11 µl, the mean being 0.07 ± 0.02 µl for 20 pools of 10 females. The blood meal volume for these pools as determined by spectrophotometry ranged from 0.03 µl to 0.08 µl, the mean being 0.06 ± 0.02 µl. The mean blood meal volume as determined by weight was significantly different ($P < 0.01$) from that determined by spectrophotometry (Fig. 3). The range in the blood meal volume as determined by spectrophotometry (0.03 µl to 0.08 µl), was lower compared to that obtained by weighing (0.01 µl to 0.11 µl). The range in blood meal volume as determined by spectrophotometry and weighing was, however, positively correlated ($r^2 = 0.48$; $P > 0.01$; SD = 0.02) (Fig. 4).

Discussion

The success of an artificial feeding method for hematophagous vectors may not only depend on the number of females that feed but also on the volume of blood taken up during feeding. Taking into account the small size of *Culicoides* species, combined with a variety factors that may influence blood feeding (Lehane, 2005), it would be advantageous if the volume of a blood meal could be taken into consideration when evaluating artificial feeding systems or when comparing oral susceptibility and vector competence studies.

Due to their small size, determining and comparing the blood meal volume of *Culicoides* remains challenging. Methods employed previously ranged from weighing of fed

and unfed females (Roberts *et al*., 1977; Fujisaki *et al*., 1987; Venter *et al*., 2005), calculating the contents of hemoglobin, nitrogen, and iron in engorged females (Leprince *et al*., 1989); using ELISAs (Foil *et al*., 1987), and sucrose labelling with radioactive sodium iodide (Muller *et al.*, 1982). Although weighing represents a simple method to determine the volume of blood taken in by a female it may, due to the elimination of access liquids during feeding, underestimate the blood meal volume (Leprince *et al*., 1989).

In the current study, spectrophotometry and weighing were compared as methods to determine the volume of blood ingested during artificial feeding. Although both methods may have underestimated the volume of blood taken in, the lower values obtained with spectrophotometry indicate that the error may significantly increase when using spectrophotometry. Although the mean blood meal volume as determined with spectrophotometry differs significantly from that obtained by weighing, the range in blood meal volumes as determined by the two methods was positively correlated. This signifies that both methods can be used to indicate the volume of blood imbibed and to evaluate factors that may influence artificial feeding. The significantly lower values obtained with spectrophotometry may be because mainly haemoglobin associated with the erythrocytes is measured, while weighing does include the plasma and other cells. It is noteworthy, however, that bluetongue, and other orbiviruses, are mostly associated with the erythrocytes (MacLachlan *et al*., 1990). The current study suggests that the burgundy pigment deposited in the walls of the abdomen during oogenesis does not influence spectrophotometry results and that the method can be used in both parous and nulliparous females.

The volume of blood ingested during feeding may not only influence the potential infection rate but may also play a role in determining egg batch size and reproductive success. Awareness of the blood meal volume takin during artificial feeding, and the factors that influence this, can be used to evaluate, and improve, artificial feeding methods and to support studies relating to the laboratory colonisation of potential vector species of

Culicoides. Previous studies reported a smaller mean egg batch in midges fed using an artificial feeding system when compared to those fed on live hosts. For example, Veronesi *et al*. (2009) reported a mean batch size of only 6.8 eggs / *C. imicola* female in midges fed through a membrane compared to batch sizes of 53 to 263 eggs reported in field collected gravid females (Braverman & Linley, 1994; Walker & Boreham, 1976; Barceló & Miranda, 2018). The mean blood meal volume ingested during artificial feeding either determined by spectrophotometry, 0.06 µl, or by weighing, 0.07 µl, may therefore underestimate the field situation.

The present results indicate a higher mean blood meal volume (0.07 µl) for *C. imicola* than that of 0.045 µl previously indicated by Venter *et al*. (2005) using weighing. Venter *et al*. (2005) used a different type of membrane (one-day-old chicken-skin) and different blood source (ovine) compared to the membrane type (Parafilm®) and blood source (bovine) used in the current study. This difference in blood meal volume highlights that membrane type (Blackwell *et al*., 1994) and blood source might influence the volume of blood imbibed by *C. imicola* during artificial feeding. It should be taken into consideration that environmental conditions e.g. seasonal variation, may influence the size of *Culicoides* (Kluiters *et al*., 2016), and that the potential influence of this on the volume of blood imbibed is largely unknown. This study illuminated the difficulty in obtaining reproducible results needed for the comparison of oral susceptibility and vector competence results in the laboratory (Wilson & Harrup, 2018).

A potential shortcoming of spectrophotometry is that a calibration curve, generated from the blood used for feeding, is needed to determine the volume of blood ingested. This implies that this method cannot be used to determine the blood meal volume in field collected females that have fed on unidentified hosts.

Conclusions

The positive correlation obtained between volume of blood determined by weighing and volume of blood determined via spectrophotometry indicates that both methods can potentially be used to evaluate factors that may influence the volume of blood taken up during artificial feeding. Despite being a more complicated method the smaller variation in results obtained by spectrophotometry may indicate a greater reproducibility when compared to weighing. Although blood meal volumes obtained by weighing where larger in comparison, spectrophotometry may represent an alternative and reproducible method that can be used to determine and compare the blood meal volume in artificially infected midges.

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Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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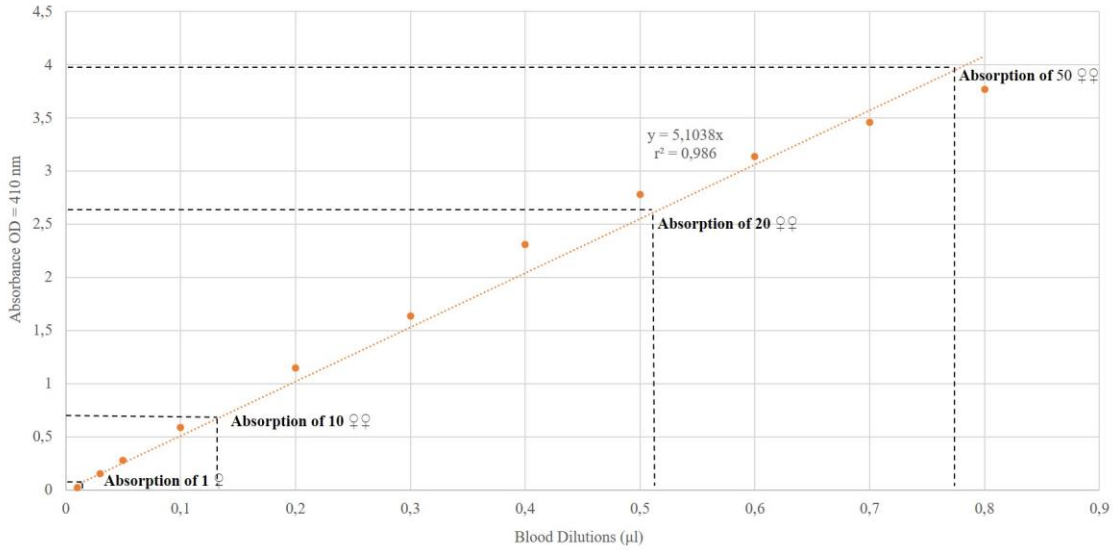

Fig. 1: Spectrophotometry absorption values, at a wavelength of 410 nm, obtained for pools of 1, 10, 20 and 50 homogenised blood engorged *Culicoides imicola* females plotted on the blood dilution range standard curve.

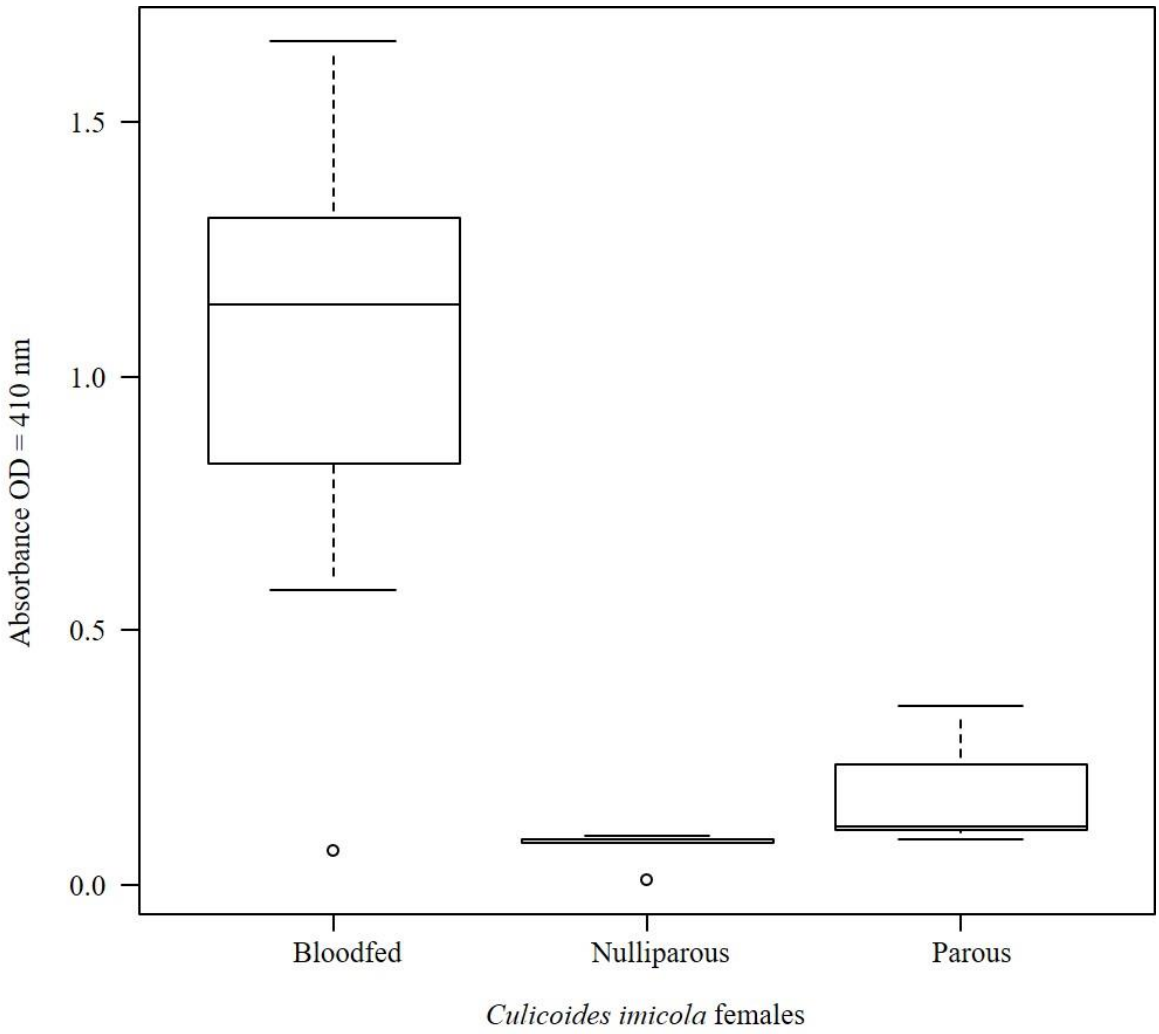

Fig 2: Spectrophotometry absorption values obtained for pools (pool size = 10) unpigmented (nulliparous), pigmented (parous) and blood-fed females. Each box shows the group median separating the 25th and 75th quartiles, capped bars indicate maximum and minimum values, circles indicating the outliers.

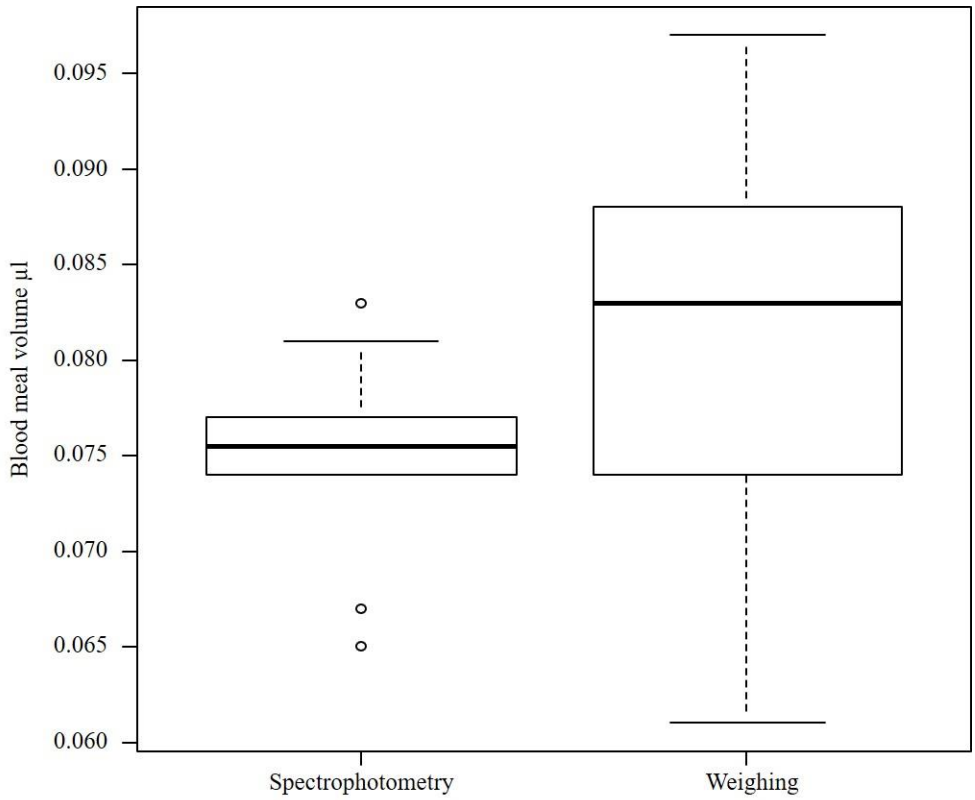

Fig. 3: Blood meal volume as determined for 20 pools of 10 females with spectrophotometry and by weighing. Each box shows the group median separating the 25th and 75th quartiles, capped bars indicate maximum and minimum values, circles indicating the outliers.

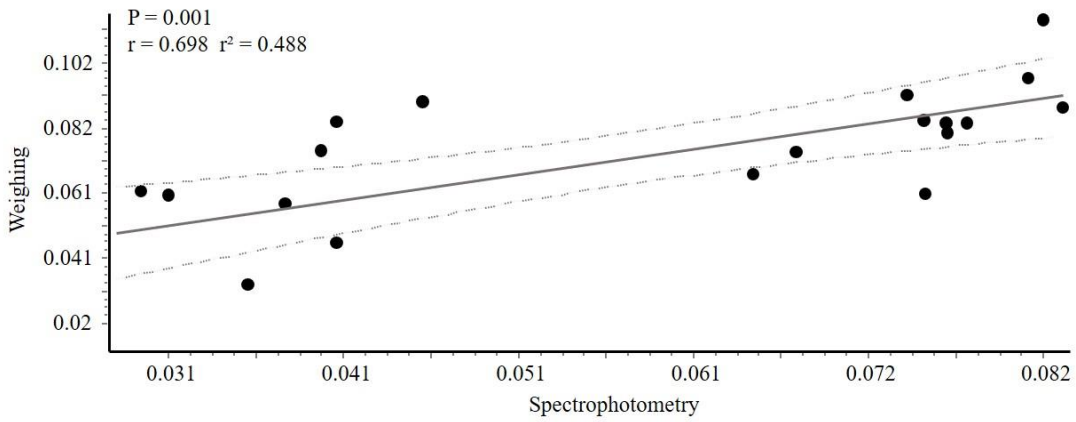

Fig. 4: Linear correlation between blood meal volume (µl) as determined with spectrophotometry and by weighing.