

An improved method for *in vitro* feeding of adult female *Dermanyssus gallinae* (poultry red mite) using Baudruche membrane (goldbeater's skin).

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Short report

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Abstract

Background *Dermanyssus gallinae*, or poultry red mite (PRM), is an important ectoparasite in laying hens, having a significant welfare and economic impact. Testing novel control compounds typically involves *in vitro* methodologies before *in vivo* assessments. Historically, *in vitro* methods have involved PRM feeding on hen blood through a membrane. The use of hen blood requires multiple procedures (bleeds) to provide sufficient material and the use of a larger species (e.g. geese), could serve as a refinement in the use of animals in research. Methods The existing *in vitro* feeding device, employing a Parafilm™ M membrane [1] and adult females was used to investigate any differences in mite feeding, egg laying and mortality when fed goose or hen blood. Effects on these parameters when PRM were fed through either; Parafilm™ M, Baudruche membrane, and a combination of these with an overlaid polyester mesh were then tested using goose blood. Results PRM fed equally well on goose or hen blood through a Parafilm™ M membrane, no significant differences in mortality of PRM fed with either blood type was demonstrated. A significant increase (t-test: $t = 3.467$, $df = 4$, $P = 0.03$) in eggs laid per fed mite when using goose blood was demonstrated. A 70% increase in PRM feeding was observed when mites were fed on goose blood through a Baudruche membrane when compared to the Parafilm™ M membrane. Addition of an overlaid polyester mesh did not improve feeding rates. A significant increase (ANOVA: $F(3, 20) = 3.193$, $P = 0.04$) in PRM egg laying was observed in mites fed on goose blood through Baudruche membrane, compared to those fed through Parafilm™ M. A mean of 1.22 (SEM \pm 0.04) eggs per fed mite was obtained using the Baudruche feeding device compared to only 0.87 (SEM \pm 0.3) eggs per fed mite using the Parafilm™ M device when neither had a polyester mesh overlay. Conclusion The *in vitro* feeding of adult female poultry red mite can be readily facilitated through the use of goose blood in feeding devices with Baudruche membrane.

Background

Infestation of laying hen houses with the poultry red mite (PRM), *Dermanyssus gallinae*, is a major animal welfare and economic problem for the egg-producing industry worldwide, costing the poultry industry in excess of €231 million per year in the EU alone [2]. Current treatment options are limited and often ineffective. PRM infestations cause serious welfare and production issues in the birds as a result of their nocturnal blood-feeding activities and potential as competent vectors for bacterial and viral pathogens [2-4]. Novel methods of controlling PRM (novel biopesticides and plant derived products; semiochemicals and growth-regulators; vaccines etc.) are typically tested using small numbers of PRM in *in vitro* efficacy assays initially, followed by field testing using large numbers of both parasites and hens [5-7]. The *in vitro* feeding devices employed in these assays contain the mites, allowing them to feed on hens' blood through a membrane such as day-old chick skin [8, 9] or artificial membranes such as Parafilm™ M [1, 10]. This methodology has technical limitations, not least the requirement for high numbers of replicates to overcome variable feeding rates when using Parafilm™ M, and issues around supply, uniformity and quality of chick skin [8,9]. In addition, to supply sufficient hen blood for these assays, multiple invasive procedures are required involving venepuncture on hens' wing veins. The aim of

this study was to investigate the use of goose blood (which can be obtained in far higher volumes per procedure than hens' blood) as a higher-welfare alternative food source for the assays and goldbeater's skin (also known as Baudruche membrane) as an alternative feeding membrane that may enable improved feeding of adult female PRM.

Methods

Blood samples

Chicken and goose whole blood samples were collected by wing vein venepuncture into a 1.5ml capacity Eppendorf tube or syringe containing heparin, respectively, (Sigma-Aldrich Co Ltd, Dorset, UK) to give a final heparin concentration of 20 units/ml blood. Hen blood from four hens was pooled for further use; individual goose bleeds were used for each *in vitro* experiment. Trials were carried out on the day of blood collection and blood was kept at room temperature (RT) until needed. Hens consisted of pullets aged approximately 20 weeks old, housed in groups in floor pens. Male geese of approximately 12 months old were used and housed in predator-proofed, enriched paddocks

Parasite material

PRM were collected and stored as previously described [11]. Briefly, mixed stage and sex *D. gallinae* were collected at three weekly intervals from a commercial egg laying unit. Prior to feeding assays, mites were stored in vented 75cm² canted tissue culture flasks (Corning, NY, USA) at RT for seven days to allow blood digestion to occur, after which they were stored at 4°C for up to 3 weeks.

In vitro feeding device

Feeding devices (Fig.1) were constructed as described previously [1, 4, 12]. For initial experiments comparing hen and goose blood as food sources the feeding membrane used was Parafilm™ M. For the experiments comparing feeding membranes, the membranes were either a 2cm x 2cm square of Parafilm™ M or Baudruche membrane (Preservation Equipment Ltd, UK) held in place by wrapping a strip of Parafilm™ M over the edges of the Baudruche membrane, around the outside of the tube, before placing the modified pipette bulb goose blood reservoir over the top (Fig. 1). In experiments to assess the impact of an overlaid polyester mesh on the feeding membranes, to improve mite attachment, a polyester mesh (Plastok Ltd, UK) of 105 µm aperture width and a 63 µm depth [10] was overlaid on the ventral aspect of the membranes used in the devices and heparinised goose blood was used as the food source.

Feeding Assays

On removing the mites from storage at 4°C, they were maintained at RT for 20 mins to allow motile mites to migrate to the cap of the flask. Only motile, adult female mites were collected from the flask caps, transferred to the feeding devices (50 mites per device) and the devices sealed. Once all feeding devices contained mites, heparinised blood was added to the devices, which were then incubated in darkness in

an incubator (Sanyo, MLR-351H) at 39°C and 85% relative humidity (RH) for three hours. Following incubation, mites were recovered from the devices and each fed mite was transferred into a single-well of a 96-well tissue culture plate (Costar, Corning, NY, USA) which was then sealed using AeraSeal™ tape (Sigma-Aldrich Co Ltd, Dorset, UK). Plates were placed into an incubator at 25°C with 85% RH and the mites observed using a stereo microscope at 3, 24, 48, and 144 hours to record mite egg laying and mortality.

For the comparison of adult female PRM feeding rates, egg laying and mortality when fed goose blood or hens' blood through a Parafilm™ M membrane, a single experiment was performed with 3 replicate assays per food source. For the comparison of adult female PRM feeding rates, egg laying and mortality when fed goose blood through either a Parafilm™ M or a Baudruche membrane (or each of these membranes overlaid with the polyester mesh) two repetitions of the experiment were performed with 3 replicate assays per device in each.

Statistical analysis

Unpaired Student's t-tests were performed on mite feeding, mortality and fecundity data for the comparison of mites fed on goose or hens' blood. An Analysis of Variance (ANOVA) was used to determine variance between the two experiments held on different days comparing the different membranes. To compare any differences between the Parafilm™ M membrane and Parafilm™ M membrane + mesh, Baudruche membrane and Baudruche + mesh, an ANOVA using Dunnett's multiple comparisons was used. To examine any difference between Parafilm™ M membrane and Parafilm™ M membrane + mesh and Baudruche membrane and Baudruche + mesh, an ANOVA using Sidak's multiple comparisons test was used. All analyses were carried out using GraphPad Prism v8 (GraphPad Software, San Diego, California USA, www.graphpad.com).

Results

Comparison of feeding between goose and hen blood

Goose and hen blood were equally successful in terms of the numbers of PRMs feeding on each blood source and survival of the mites following feeding (Table 1). There was a 26% increase in total egg production in mites fed on goose blood compared to hen blood and the number of eggs laid per fed mite was significantly higher in those mites fed on goose blood (t-test: $t=3.467$, $df=4$, $P=0.03$).

Comparison of PRM feeding on goose blood through Parafilm™ M, Baudruche membranes and in those membranes overlaid with polyester mesh

No statistically significant differences were demonstrated within the same treatment groups in the two repetitions of the experiment in either feeding, egg laying, progeny per fed mite or mortality and therefore

replicates from the two repetitions of the experiment were combined for further analysis. Comparison of feeding rates, fecundity and mortality between PRM fed with goose blood through the different membranes is shown in Table 2. The Parafilm™ M membrane in one of the feeding devices developed a split during the incubation period, which is common with this type of membrane, whereas none of the Baudruche membranes failed. No feeding occurred in the failed device and so this replicate was not included in the analysis. A 70% increase in the mean number of PRM feeding was observed when the mites were fed on goose blood through a Baudruche membrane compared to a Parafilm™ M membrane (Fig. 2, Table 2) though, because of the high levels of variability in feeding levels of mites feeding through a Parafilm™ M membrane, this increase was not statistically significant ($P = 0.1$). Addition of an overlaid polyester mesh did not improve feeding rates on either membrane and a significant (ANOVA: $F_{(3,20)} = 3.1$, $P = 0.04$) decrease in feeding was demonstrated (Table 2) in the devices containing Baudruche + mesh compared to Baudruche membrane alone. Mites which had fed through Baudruche membrane (without overlaid mesh) produced double the number of eggs of those fed through a Parafilm™ M membrane (ANOVA: $F_{(3,20)} = 3.193$, $P = 0.04$) over the course of the experiment (Table 2; Fig. 2).

Discussion

Here we have demonstrated that goose blood represents an excellent food source for laboratory maintained PRMs and that Baudruche membrane is a superior feeding membrane to the traditionally used Parafilm™ M for *in vitro* feeding assays. Geese are well suited as blood donor animals as their size means they can donate more blood per procedure than hens (approx. 20 fold). Generally, blood collected from individual hens is pooled for use in feeding assays and the amount collected can be limiting in terms of replicates and/or size of experiment. A statistically significant increase in eggs laid per fed mite was demonstrated using goose blood compared to hen blood in the initial experiment using the Parafilm™ M only device.

To our knowledge, this is the first published report on the use of Baudruche membrane in the feeding of *D. gallinae*, although it has been used for tick feeding in conjunction with silicone as recently as 2019 [13] and for mosquitoes as early as 1964 [14]. Although chick skin has been used successfully to allow *in vitro* feeding of PRM [5, 8, 9] availability and ease of use of artificial membranes has led to their more frequent use. Membranes such as Parafilm™ M [1] or Nescofilm coated with a hen skin extract [10] have been employed and can work well. However, Parafilm™ M membranes often fail due to the need to stretch the membrane to make it thin enough for mites to feed successfully and Nescofilm is no longer commercially available. Membrane failure makes recovery of fed mites from the device difficult and can lead to misleading mortality data [6] and the rate of device failure and highly variable feeding rates requires additional replicates with the associated increased invasive sampling of hens. Therefore, a more reliable *in vitro* feeding device increases hen welfare in terms of refinement in number of procedures required for successful data collection.

Krull et al., 2017 [15] suggested that thin membranes for the feeding of tick larvae and nymphs could be obtained using Baudruche membrane coated in silicone, or by using Baudruche membrane alone. As

previous attempts at using silicone with lens tissue as described in [16] had not resulted in PRM feeding in our laboratory (data not shown) it was hoped that the reported high tensile strength of the Baudruche membrane alone would suffice and that it would be thin enough to allow the mites to feed, without damaging the membrane. The addition of a mesh support was successfully used in previous tick studies [14,16] which resulted in enhanced attachment times of ticks to membranes. It was decided to test a mesh that had already demonstrated utility in the feeding of PRM [11] to determine if attachment was enhanced. No difference was observed in feeding rates or egg laying with the Parafilm™ M only membrane or the Parafilm™ M combined with mesh. A significant decrease in feeding was demonstrated between the Baudruche membrane combined with mesh in comparison to Baudruche membrane alone. This might be attributed to a lack of tension in the Baudruche membrane in this system and the subsequent difficulty for the mites trying to attach through both mesh and membrane compared to membrane alone. Greater variability was observed in both feeding rates and in the number of eggs laid per fed mite when using only Parafilm™ M when compared to Parafilm™ M used in conjunction with mesh, whereas neither feeding rates or eggs laid per mite were affected by the addition of the mesh. In addition, no membrane failures were observed when using the Parafilm™ M with the overlaid mesh.

It was observed in this study that mites that had fed on goose blood through the Baudruche membrane were fully engorged when compared to those that had fed through the Parafilm™ M membrane. This repletion level may explain the differences in the trend of increased egg production in the Baudruche fed mites. Therefore, the textured surface of the Baudruche membrane may facilitate easier mite attachment, enabling them to feed to repletion, when compared to the smooth Parafilm™ M membrane. The robust performance, availability and good feeding rates makes Baudruche membrane a useful alternative to chick skin and artificial membranes for use in PRM feeding.

Conclusion

When applied to novel interventions being delivered to PRMs *via* hen blood (e.g. novel vaccines and systemic acaricides) *in vitro* studies have previously suffered from highly variable mite feeding rates and high background mortality of mites when using the *in vitro* feeding system [5,6]. A more reliable *in vitro* feeding device reduces the necessity of higher replicates and therefore the volume of blood required, leading to a refinement in animal procedures.

A more reliable *in vitro* feeding method is ideal for initial screening of new control compounds and for those studies that don't require on-hen testing e.g. RNAi studies. Here, we have demonstrated the potential of using Baudruche membrane for *in vitro* feeding of adult poultry red mites with further studies planned to examine its use for the other hematophagous life stages of the parasite.

Declarations

Ethical Approval

All experimental procedures described here were approved by the Moredun Animal Welfare and Ethical Review Body. Goose bleeds were conducted under the legislation of UK Home Office Project License (reference P33D3D364 for geese and P46F495BD for hens) and in accordance with the Animals (Scientific Procedures) Act of 1986

Consent for Publication

Not applicable.

Availability of data

The datasets supporting the conclusions of this article are provided within the article and can be acquired from the corresponding author on request.

Competing interests

The authors state they have no competing interests.

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Author contributions

Conceived and designed studies: FGN, JB. Performed experiments: FGN, JB, KB. Analysed data: FGN, JB. Statistical analysis: FGN. Prepared manuscript: FGN, JB, SB, AJN. All authors revised and approved the final version of the manuscript.

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Tables

Table 1. Poultry red mite feeding, egg laying and cumulative mortality 144 hours after the mites had been fed with hen or goose blood. Mean numbers of mites and eggs are shown (n=3) with standard error of the mean (SEM).

Test parameter	Hen blood	Goose blood	Unpaired t-test
Number of mites feeding	19.0 ± 3.5	19.0 ± 5.5	P = 0.96
Total number of eggs	28.6 ± 7.7	35.3 ± 9.4	P = 0.61
Eggs laid per fed mite	1.56 ± 0.08	1.87 ± 0.03	P = 0.03
Number of dead adult mites after 144h	1.00 ± 0.6	1.00 ± 0.6	P = >0.99

Table 2. Comparison between numbers of adult female poultry red mites feeding, egg laying, eggs laid per fed mite and mortality using goose blood as a food source and either Parafilm™ M, Baudruche membrane or a combination of either with an overlaid polyester mesh. Mean values are shown with SEM, n=6 except where stated otherwise.

Test Parameter	Parafilm ^a	Baudruche	Parafilm/mesh	Baudruche/mesh
<i>Number of mites feeding</i>	16.0 ± 3.6	25.8 ± 4.2	17.5 ± 3.9	13.2 ± 2.7
<i>Total number of eggs laid</i>	16.8 ± 3.7	33.7 ± 5.8	20.5 ± 3.9	17.1 ± 3.8
<i>Eggs/ fed mite</i>	0.87 ± 0.3	1.22 ± 0.04	1.21 ± 0.1	1.27 ± 0.07
<i>Number of dead adult mites after 144h</i>	0.4 ± 0.24	1.3 ± 0.6	0.7 ± 0.21	0.3 ± 0.21

^a n = 5 due to failure of one device, which led to zero feeding.

Figures

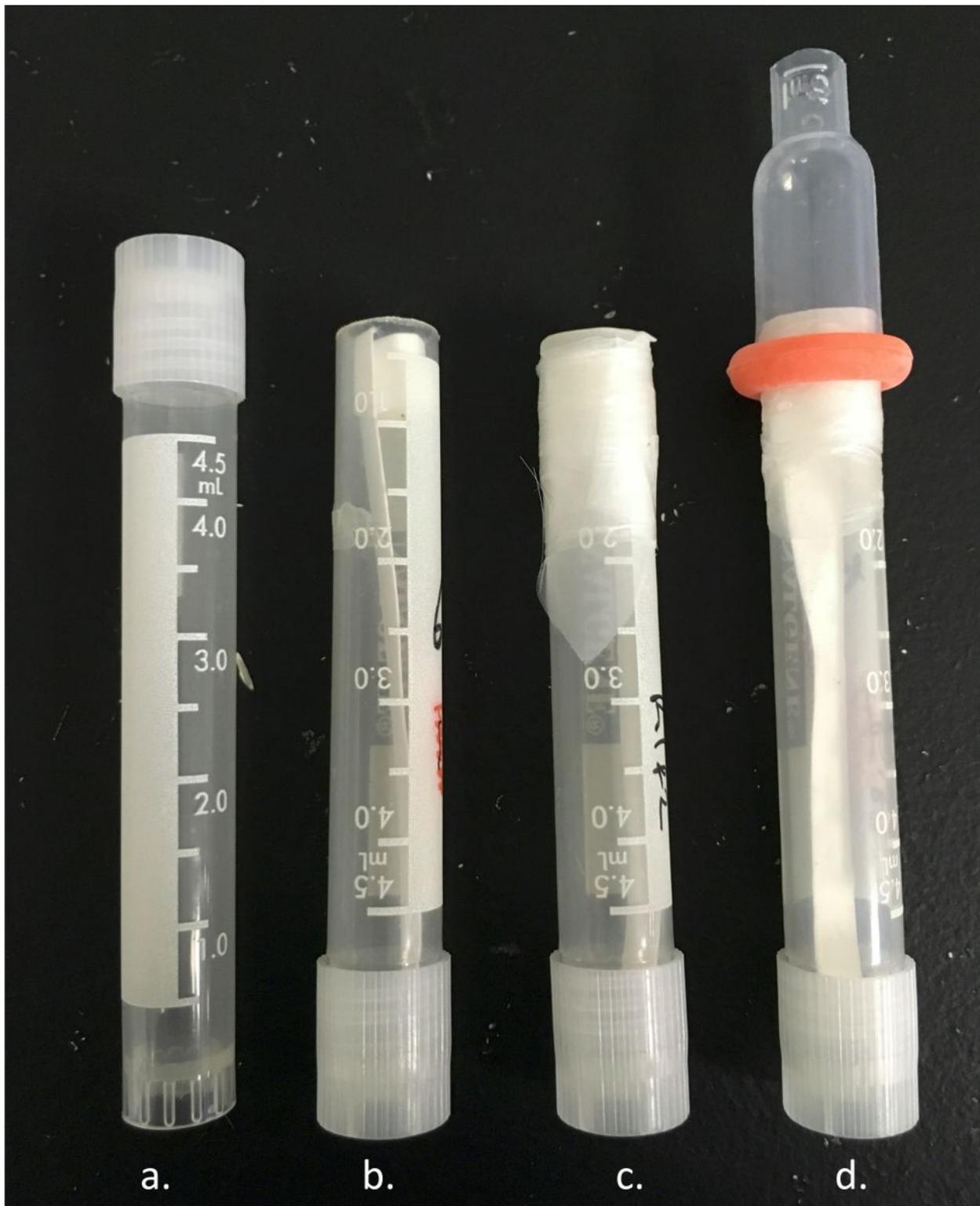


Figure 1

Sequence of construction of In vitro feeding device for poultry red mites. The closed end of a 5ml cryotube (A) is cut off, the tube is inverted and a piece of filter paper inserted (B). Mites are added to the tube and a square of mesh, Baudruche membrane or Parafilm™ M is placed over the cut off end and held in place by a strip of Parafilm™ M stretched around the outside of the tube (C). A cut down pastette bulb is then placed over the membrane and a watertight seal is obtained by using a castration ring (D). Blood

is placed inside the pastette bulb and the device is placed in an incubator in the dark to allow mite feeding to commence.

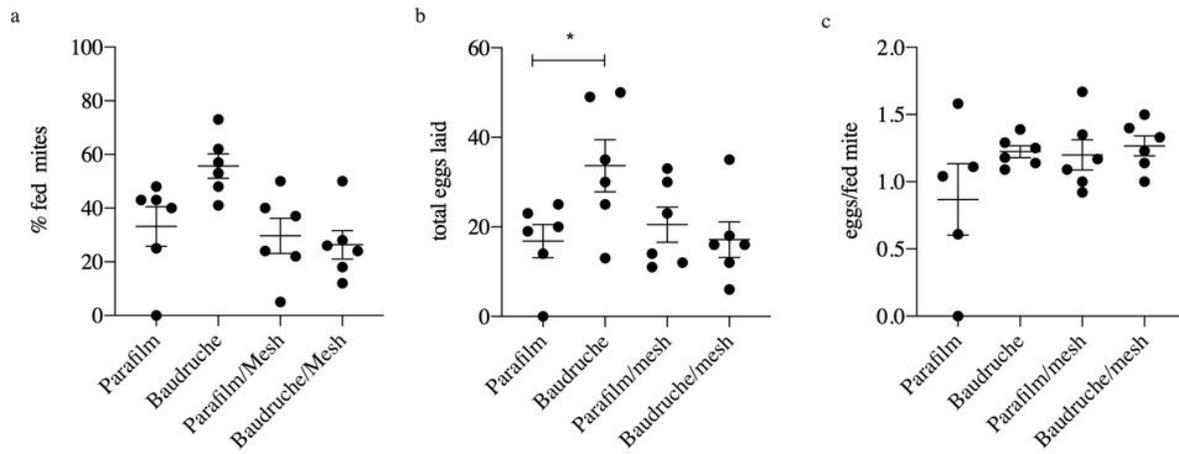


Figure 2

Feeding and egg laying of poultry red mites fed on goose blood through different membranes. Panel A: Feeding success rates of mites through different membranes; Panel B: Total eggs laid over the course of the experiment; Panel C: Eggs laid per fed mite when fed on goose blood through different membranes. Bars demonstrate mean and SEM (n = 6). A significant increase in the number of eggs laid by mites feeding on goose blood through Baudruche membrane without an overlaid polyester mesh was demonstrated (indicated with an asterisk on Panel B: ANOVA: $F(3, 20) = 3.193$, $P = 0.04$).