

This study investigates the role of air bubbles in the underwater adhesion of the hairy adhesive pads of ladybug beetles. The authors approached this topic both experimentally and theoretically. The pad adhesion of real beetles was measured using a sophisticated indentation methodology both on hydrophilic and -phobic substrates comparing cases with and without an air bubble trapped between the hairs of single pads. On hydrophilic glass, underwater adhesion is generally lower than in air, and removal of the bubble leads only to a slight further reduction of adhesion. On hydrophobic treated glass, adhesion is relatively high and unaffected by wetting state and bubble removal. The authors furthermore developed a hybrid analytical-numerical model of the capillary adhesion of a hairy insect pad with and without trapped bubble. This model is able to qualitatively approximately match the trends of the experimentally measured adhesion data for the different conditions. The authors use this model for further predictions of the effects of variations of parameters such as bubble volume and hair size on adhesion. Finally, the authors conclude that the bubble is of relatively low and only indirect importance (i.e. the bubble enables on hydrophilic substrates more hairs to form contact in a quasi-dry state) in beetle adhesion.

The manuscript is generally structured logically, written clearly, and the experimental and theoretical measurements were done systematically and mostly thoroughly. The developed theoretical model of the underwater capillary adhesion of insects is novel in the field of bioadhesion, and the aim to obtain a better understanding of underwater insect attachment very timely. Therefore, I believe that this work can form a novel and relevant contribution to *Journal of Experimental Biology*, as well as to the field of bioadhesion. That being said, I also have several substantial concerns and questions that need to be addressed before considering this article for publication. My major points of concern are (see specific comments for details and suggestions on improvement):

1. Study focus and manuscript structure

While the study is timely, novel, and relevant, the study focus could be worked out more clearly in the manuscript. Do you aim to explain your experimentally measured data using a theoretical model, or to develop a novel theoretical model and validate it through experiments? The core of both story versions is the same, but the framing differs.

2. Experimental approach

The used methodology is neat, but some aspects could be described more clearly and extensively.

3. Data analysis and statistics

I believe that the available data could be analysed more extensively and using a different statistical approach, which would help in the interpretation of the experimental results. Also, some aspects are insufficiently described.

4. Theoretical modelling

While the developed theoretical model roughly agrees with the experimental results in a qualitative manner, I have several concerns. The model (and experimental data) could be validated more thoroughly. Did you do a sensitivity analysis? The discussion should address more explicitly which assumptions are fair, and which may have affected your conclusions.

5. Gecko discussion

Due to several reasons I suggest removing this section and focusing instead on model validation and application of the model to insect attachment.

6. Wording and clarity

Some statements are not or only partially clear to me and require revision.

I did my best to evaluate this manuscript fairly and rigorously, and look forward to a reply by the authors. Please also contact me if any of my comments is unclear. Below, I list my more specific comments, classified by the main points of concern, and into other major, moderate, and minor comments. The line numbers refer to the document which I attached to this mail (“JEXBIO-2021-242852v1-Endlein_comment.pdf”). In the document, I also corrected some minor typos and suggested some rephrasing.

Yours sincerely,

Julian K.A. Langowski

Study focus and manuscript structure

1. P. 6, L. 5-6: “The goals of this study to investigate “if an air bubble is necessary for adhesion and what, if any, contribution it has to the adhesive force” and to develop a theoretical model that can explain the capillary underwater attachment of insects are valuable to the field of bioadhesion. However, I am a bit confused by the implementation of these goals in the manuscript. It is not clear to me if the authors aim to tell a story where they (a) explain their experimental findings using the theoretical model (which the overall structure and also the introduction seem to indicate), or (b) develop a novel theoretical model and validate it using experimental measurements (as written in the discussion “the experiments don’t show the predicted 2.6 times increase in underwater adhesion relative to that in air on the hydrophobic PFOTS-coated) surface.”. Either storyline is fine by me, but I believe that a revision of the manuscript could help to clarify the focus of this study. If the authors decide to follow storyline (b) I suggest highlighting the novelty of the developed model more strongly. I also invite the editor to comment on this.

The story tries to primarily present our experimental finding about the relevance of bubble for underwater adhesion, with the model being used to support the results (case a, above). However, we believe the model, albeit its simplicity, could be relevant for other systems as well. For this reason we also included the part on the gecko adhesion in the discussion (see p.9,ln.927-997)

Similarly, I find the introduction of the model too abrupt. The introduction section mentions the modelling aspect only shortly and then smoothly fades into the experimental part. The following switch to the theoretical part seems quite sudden (for example, the relevance of sections 3.4 and 3.5 only became clear to me on P. 20, L. 4-5, and the link between these sections and the experimental data is still not entirely clear to me), and I suggest to introduce the reader more gently to the modelling part (especially taking into account that this manuscript is submitted to the Journal of Experimental Biology).

The text has been updated to improve the introduction and motivation of the modelling sections (see p.2,ln.151-206; p.4,ln.432-437)

The main question (what contribution does the bubble have to the adhesive force?) could be addressed even more explicitly. Throughout the manuscript, I could not find any quantitative mentioning of the actual bubble contribution to adhesion.

The bubble’s contribution is less than 3% (mentioned explicitly in p.7,ln.756, and p.10,ln.1034). Also, see Fig. 7.

Furthermore, I invite the authors to embed the experimental and modelling results in a wider context.

- Before adding a rather speculative discussion on the role of capillary adhesion (see also comment 24), I first would like to see a clean validation of the developed model

and experimental data. How well do the measured force agree with literature data? This could even be a distinct section of the discussion. Could you provide a validation of the theoretical model using a simplified synthetic adhesive setting?

Sensitivity analysis has been included in supplementary material to validate the model and discussed briefly (p.8,ln.894-900 and suppl. section S5). Using a synthetic structure is part of another project, and unfortunately goes beyond the scope of the present study.

- What is the biological relevance of the results shown in Figures 5, 7, and 8? Which ranges on the x-axes of these figures are biological meaningful?

Fig 5,7 and 8 have been updated and show now values in dimensional units to help with the interpretation in a biological context.

Can you tentatively explain morphology and secretory systems of hairy insect pads based on these results?

We went rather the other way round: the model relies on previously reported information on the nature of the insect's attachment system (such as hair geometry, oily secretions, fluid volume) to arrive at the predictions.

Such discussion points would fit the overall manuscript more closely than the aforementioned discussion of gecko adhesion.

Experimental approach

2. P. 6, L. 5-6: "Beam deflection was calibrated using 4 different known weights to get the corresponding force" – To my knowledge this setup has not been described in detail in foregoing publications. Therefore, it would be good to describe the setup performance in more detail in the supplement to address open question such as: How much did the beam deflect and did you correct for this in the adhesion measurements? How linear was the system, and which force resolution does it have?

A few additional details such as force resolution, information on calibration weights have been included. (p.3, ln.259-264)

3. P. 6, L. 12-13: "bringing the insect in contact with the substrate from the top." – What step size was possible with the z-stage?

The z-piezo stage was moved with a step size of 75 nm with a resolution of 3 nm (p.3,ln.270-273), significantly lower than the length of the insect's hairs (roughly 40 μm).

4. P. 6, L. 23: "averaged to 512 points per motion step." – I do not understand this statement. Does it mean that motion happened stepwise at roughly 2 Hz?

The acquired force data was averaged to 512 points per step to get a smooth data (p.3,ln.287)

5. P. 6, L. 26 – P. 7, L. 4: I appreciate this movement protocol to mimic the natural movement of the beetle legs. However, I am wondering if the parameter values (100 μm sliding, 10 μm 2nd indentation, 1 s pause) were chosen based on some earlier published works or arbitrarily? Can you elaborate?

The combination of 100 μm sliding, 10 μm indentation and 1 s pause was used primarily to ensure good contact of the pad with the surface (see plot in Figure 1). The implemented parameter values were arrived at by systematically varying them one at a time.

6. P. 7, L. 11-12: “was first degassed separately in a vacuum chamber at 10 mbar pressure for 3 hours and then pipetted into the holder immediately” – I have two questions regarding this sentence.

- 1) Deaeration may have had an effect on the surface tension of the water. Albeit this effect probably was small (see, for example, Karagianni, M., & Avranas, A. (2009). *The effect of deaeration on the surface tension of water and some other liquids. Colloids and Surfaces A: Physicochemical and Engineering Aspects*, 335(1-3), 168–173. doi:10.1016/j.colsurfa.2008.11.002), a simple comparison of the contact angles of regular and degassed water would help to remove any doubts about the assumed surface tension of degassed water.

Contact angle values for de-gassed water were very similar to that of normal water. (supplementary S2, p.2,ln.12)

- 2) Did degassing immediately remove the bubble on the beetle pad, or did the bubble slowly get absorbed? The experimental protocol could get clarified regarding this question.

The bubble was removed within 5 mins after beetle immersion, via diffusion (p.3,ln.236-244)

7. P. 7, L. 13-14: “was brought into contact with the clean dry surface 10 times repeatedly to ensure the hairs are free of any contaminating particles.” – While this procedure certainly helped to reduce contamination, I am wondering about possible effects on the available adhesive liquid volume. In *Nauphoeta cinerea*, the footprint volume left behind decreases drastically within the first 7 steps (Dirks, J.-H. & Federle, W. *Mechanisms of fluid production in smooth adhesive pads of insects Journal of The Royal Society Interface*, The Royal Society, 2011, 8, 952960). Did something similar occur here? If so, please discuss accordingly. Also, I am wondering if these 10 cleaning steps were done manually or using the experiment setup? If the latter, could you provide in the supplement more information on the exact settings?

We used the same settings for the adhesion test as well as for the cleaning steps. (p.3,ln.243). The repeated measurements do increase adhesion by roughly 10% on average during the 5

tests, likely due to the gradual loss of fluid which increases capillary force. This however can't be eliminated unfortunately, and thus remains as a source of standard deviation. The linear-mixed model results reveal a relatively insignificant influence of repeats on adhesion, thus the effect of loss of fluid can be safely disregarded (supplementary Table S3).

Data analysis and statistics

8. P. 7, L. 6-7: "side view imaging was used to visually aid orienting the pad with the substrate before a test" – I suggest to extract from these recordings information on alignment variation (e.g. angle between horizontal and pad surface) and use this additional data as random parameter for correction in the statistical analysis. From the inset in Figure 1, I deem this possible.

The pad tilt angles have been extracted and fall within a range of $21 \pm 6^\circ$. The linear mixed-effect model thus reveals no significant influence of the angle on adhesion (supplementary Table S3).

Similarly, information on the contact area shape (e.g. aspect ratio) could help to motivate the assumption of circular contact areas in the theoretical model. How well does that assumption reflect reality, and what does it say about model validity?

Based on contact images of the pad in air, median eccentricity of the hair tips was 0.04, justifying the circular contact assumption. (p.4,ln.433-436)

9. P. 7, L. 16: "were averaged to avoid pseudo-replication during data analysis" – How much variation occurred throughout these 5 measurements per individual per contact mode combination? See also comment 12.

No more than 10% variation in adhesion was within the 5 measurements, which is less than the deviations seen between insects (12-24% depending on contact mode/surface). As mentioned in a previous comment, the linear-mixed model results revealed an insignificant influence of repeats on adhesion (supplementary Table S3).

10. P. 8, L. 2-5: While uploading the script on github is highly appreciated, mentioning some of the key aspects (e.g. did you correct for non-infinite beam stiffness?, how was contact area computed?) would be useful for the reader, especially if not experienced with Python.

Since the contact area is not used in any of the analysis, we have chosen not to elaborate on the image processing routine (also see response to comment #12 below).

11. P. 9, L. 5-8: This correction for additional capillary forces due to the contact line between water surface and beetle leg is appreciated! However, the description of the protocol could be clearer. Do I understand correctly that you measured the 'background' force curve for

each single trial? Or once per individual? How did you align the background curve with the real-contact-force curve? How large was the additional capillary force relative to the pad adhesion? How much did the background curves vary between individuals/trials? A more extensive explanation of this protocol in the supplement could answer many of these questions.

“Background” force curve was obtained once per individual. The same movement settings as a typical adhesion test was used here, which means the background subtraction could be performed directly by matching the time value of data points. The additional capillary force was not more than 50 uN, significantly lower than the adhesion force of the pad. Proper subtraction was ensured by the presence of a “flat” response in the regions of the force curve where the pad makes no contact (i.e. during initial approach and after pull-off during retraction) (p.3,ln.332-342)

12. P. 9, L. 9-11: “Data sets were compared for statistical differences using two-way ANOVA analysis, with contact mode and substrate chemistry as the categorical variables and adhesion force as the dependant variable” – So you did not use the contact area data in the statistical analysis? If so, why not? This would be a straightforward way to correct the measured forces for variations in effective contact area.

Due to the very different image contrast conditions between measurements done in air and underwater (see supplementary video S2), it was not possible to maintain a consistent image processing protocol to extract contact areas. The extracted areas were usually overestimated for contact in air, while underestimated for contact underwater. Thus we unfortunately couldn’t use the contact area to make any statistical comparison between the different contact modes.

I suggest using a linear mixed-effect model (see, for example, *Langowski, J. K. A.; Rummenie, A.; Pieters, R. P.; Kovalev, A.; Gorb, S. N. & van Leeuwen, J. L. Estimating the maximum attachment performance of tree frogs on rough substrates Bioinspiration & Biomimetics, 2019, 14, 025001*) and correcting for contact area variations as well as beetle identity (see comment 9) in order to make full use of the available data.

As recommended, linear mixed-effect model results have been included in the supplementary (section S3).

13. P. 11, L. 1-2: “Apart from the three depicted contact modes, we observed an additional fourth mode which occurred in roughly 25% of our underwater experiments using degassed water.”

This is an interesting observation! Were these datapoints excluded from the ‘underwater: no bubble’ data (could this explain the relatively small variation of the according boxplots in fig. 1)? If so, this should be specified.

Yes, these data points were not included in either the plot or for statistical analysis. They are reported here independent of the primary results. The number of data points for each “box” of Figure 2 remains the same, and should not influence the variances. (p.4,ln.387)

I am a bit confused regarding supplemental video 2, which shows such a bad contact, right? There, I still see a recorded force, which disagrees with the statement on P. 12, L. 2. What happened here?

Note that negative values represent attractive forces (adhesion) in the plot. The recorded force you see in Movie 2 corresponds to the typical loading and shearing cycle when the pad is brought into contact. However, the final retraction step (green shaded) shows no negative pull off force, indicating there is no adhesion.

Theoretical modelling

14. P. 2, L. 22-24: “[...] discoidal shaped seta show larger pull-off forces than spatula shaped or pointed setae, illustrating the role of hair geometry in adhesion” – How does this observation fit with the capillary adhesion model developed here? Is the model able to reflect this observation? If not, why?

The model is more suited for discoidal hairs rather than spatula hairs since we assume the hair tip to be circular and that the secretory fluid remains pinned at the hair tip. The flat circular shape and rim of the discoidal shaped hair should make the above assumptions reasonable. This is why our experiments were done on male ladybugs, which have predominantly discoidal hairs, so as to make a meaningful comparison with our model predictions. For the case of spatula hairs (in female ladybugs), the elastic deformation of the tip would further increase the contact area, which will influence its adhesion. Elastic effects are however not accounted for in our model to keep things simple. In this case, an elastocapillary model would be more appropriate (Gernay et.al. 2016). (see p.4,ln.432-436)

15. P. 13, L. 4-5: “The hairs and the pad were assumed to be perfectly rigid, for simplicity.” –

I agree that assumptions have to be made for simplicity in modelling biological systems. However, it has been shown repeatedly that elastocapillary effects can be important in the adhesion of insects (e.g. Gernay, S.; Federle, W.; Lambert, P. & Gilet, T. *Elasto-capillarity in insect fibrillar adhesion* *Journal of the Royal Society Interface*, 2016, 13, 20160371; <https://www.sciencedirect.com/science/article/abs/pii/S2214574518300993?via%3Dihub>) and other animals (Butt, H.-J.; Barnes, W. J. P.; del Campo, A.; Kappl, M. & Schönfeld, F. *Capillary forces between soft, elastic spheres* *Soft Matter*, 2010, 6, 5930-5936). Therefore, the implications of neglecting such effects should at least be discussed, especially when presenting a novel adhesion model to explain ladybug beetle underwater attachment.

See above comment for #14. The shortcomings for the model due to its various assumptions (including that of stiff hairs) is discussed briefly in p.8,ln.880-901.

16. Equation 1: The force of individual hairs " f " is at the core of your model, and should therefore be described mathematically not only in the supplement but also in the main text.

Included as equation 3 (p.5)

17. P. 15, L. 15-21 and Table 1: It is repeatedly mentioned that 'typical' values were used, so I assume that the literature was screened for such values. Please provide the according references in Table 1 for the different parameter values, or explain how else these values were determined.

The parameters and relevant references have been updated (see Table 1 and p.5,ln.546-554)

Also, did you perform a sensitivity study to test for effects of potential inaccuracies on your conclusions? How much do your conclusions rely on the exact values chosen? Related to comment 1, addressing these questions is crucial for full validation of the developed model.

Sensitivity analysis of the model is included in the supplementary file (section S5) and briefly discussed in p.8,ln.894-901)

18. P. 17, L. 5: Is the assumption of pinning valid for ladybug hair attachment? Can you refer to any literature to strengthen your point?

Based on the discoidal hair's geometry, mechanical pinning of the liquid around its rim can be expected. As far as we are aware, however, there are no studies published which directly validate this assumption for the insect's hair.

19. P. 19, L. 3-4: "Additionally, the interfacial tension of the oily fluid underwater (f_w) is twice that of in air (f_a)."

– A literature reference would help to strengthen this statement.

Reference included. (p.5,ln.549)

20. P. 23, L. 1-2: "capillary force due to a single fluid bridge decreases due to its smaller size and "self-similar" scaling assumption ($f \propto D_h$)," – As shown in equation S1 in the supplement, the surface tension and laplace term scale differently with hair size (length- vs. area-scaling), which should result in a variation of relative contribution of these two effects with changing hair size. Therefore, the statement of self-similar scaling may not be valid, and this assumption should be checked and discussed accordingly.

The total contact area is kept fixed for this particular analysis and thus, only the length-scaling term of the capillary force is relevant.

21. P. 25, L. 1-2: "[...] qualitative trend is consistent for both hydrophilic and hydrophobic substrates in air and underwater." – By plotting simulated over experimentally measured data, you could further characterise this trend and possibly strengthen your conclusions.

If you are referring to comparing the force-distance curves, we are not sure what additional characterizations are possible in this case. Since the assumed detachment mechanism in the model (simultaneous contact-loss) vs the experimental detachment mechanism (a sequential contact loss of hairs) are quite different, we believe making a comparison between the force-distance curves would not be meaningful. The adhesion values have been compared however in Figure 2.

22. P. 24, L. 3-4: “Predictions of the ladybug’s adhesion from the capillary bridge model agree with our experimental results” – I tend to disagree. With deviations of up to several 100%, the model at best qualitatively agrees with the experimental data.

Reworded: “Predictions of the ladybug’s adhesion from the capillary bridge model agree qualitatively with our experimental results” (p.8,ln.859)

23. P. 24, L. 26: “Thus, it’s not surprising that the model overestimates the adhesion forces” – This is true for most but not all substrate conditions. For ‘underwater: no bubble’ on glass, the model seems to slightly underestimate the experimentally measured force. Can you explain that?

With the updated model parameters, in particular the interfacial tension value of the secretion fluid to be same as that of n-hexadecane, we no longer see the pointed out discrepancy. Previous values corresponded to low carbon alkane such as hexane, which are quite volatile. Hexadecane, in comparison, is a more reasonable choice, since the insect’s secretions are known to primarily contain long chain hydrocarbons.

Gecko discussion

24. P. 25, L. 17-P. 26, L. 19: This tentative discussion on the role capillary forces in gecko adhesion appears critical to me due to several reasons:

- 1) Most importantly, as stated in also in comment 1, this part of the discussion appears premature. Before applying the model to other animal groups, it should be validated for insects first. Do the measured and predicted forces even agree with values reported for insects in the literature?

Experimental measured forces are of the same order of magnitude as literature values reported for dock beetles (Bullock et. al, J Exp Biol 2009, 212, Pt 12, 1876-88). For model validation, sensitivity analysis has been included (supplementary section S5). Simulation results for a single capillary bridge also agree with previously reported simulations and experiments (Figure 4 in present study; De Souza et. al. Langmuir 2008 Vol. 24 Issue 16 Pages 8813-8820; De Souza et. al., Langmuir 2008 Vol. 24 Issue 4 Pages 1391-1396)

- 2) Figure 9 compares shear forces (i.e. friction) in geckos with pure adhesion in insects. This comparison is questionable due to different nature of the involved phenomena. The developed theoretical model to my knowledge does not even compute contact forces under shear loading.

That is true, however friction forces in animals typically correlated to the normal adhesion force, thus using the data for a qualitative comparison should be reasonable. (see for e.g. Endlein et. al., PLOS ONE 10(11): e0141269 for ants)

- 3) Are liquid volumes of gecko and insect pads even in the same order of magnitude, and is the theoretical model applicable to the supposedly lower volumes on gecko setae?

The liquid volume of gecko is 3 orders of magnitude lower than the insects' case, due to their smaller hair size. For the assumed fluid volume for the gecko (~0.004 fL), the fluid thickness should be ~ 10 nm, which is close to the range where vdW forces get weak. Application of the capillary model should thus be reasonable, as long as the layer forms a liquid meniscus.

- 4) There is strong evidence for involvement of van der Waals forces in gecko adhesion.

For example, gecko setae adhesion is sensitive to sub-surface energy variations (*Loskill, P.; Puthoff, J.; Wilkinson, M.; Mecke, K.; Jacobs, K. & Autumn, K. Macroscale adhesion of gecko setae reflects nanoscale differences in subsurface composition Journal of The Royal Society Interface, 2013, 10, 1-8*), which cannot be explained with capillary theory. Therefore, this discussion—if left in the manuscript (see below)—should provide a full account of evidence for and against the different adhesion mechanisms.

The study on sub-surface effects on the adhesion is quite interesting. But this should not invalidate the capillary theory, because one may expect that such sub-surface features to influence the contact angle of the liquid via similar interactions.

In light of recent evidence of the polar interaction in gecko adhesion as a consequence of its phospholipid layer (Singla et al., Science Advances 19 May 2021: Vol. 7, no. 21, eabd9410), we believe that reinterpretation of previous adhesion data by considering a capillary effect of this layer should be relevant to the debate of vdW forces vs capillary mediated adhesion. Thus we have decided to keep this section.

Finally, this part of the discussion comes rather surprising and does not really follow from the main storyline. The introduction only mentions “with regards to understanding adhesion in other animals”. Anyhow, based on this list of reasons, I suggest removing this part of the discussion and instead focussing on model validation and application to insect attachment. If the authors decide otherwise, the ‘gecko excursion’ needs to be embedded more smoothly in the manuscript.

The gecko discussion has been reworded to integrate better with the story. (p.9,ln.926-997). We have however refrained from mentioning it in the introduction section, since this is not the main message of the paper, but simply showcases an interesting application of the presented model. By this, we hope to motivate the community into further investigating the underlying details of gecko adhesion.

Wording and clarity

25. Title: “controls” – I suggest replacing this word with “determines” or similar, since “controls” implies the possibility of actively varying underwater adhesion by the beetles, which is not shown. **Changed to “determines”**
26. P. 3, L. 13: “[...] can attach quite well [...]” – Please quantitatively specify this statement. **Quantifying it seems unnecessary. Reworded to “can attach to surfaces underwater”.(p.1,ln.100)**
27. P. 3, L. 9-10: “One relatively simple approach” – I suggest either removing “relatively simple” or explaining why the bubble-approach is simpler than other ones. To me the simplicity of this mechanism is not obvious. **Removed. (p.1,ln.95)**
28. P. 5, L. 21: “doesn’t” – Such contractions occur repeatedly throughout the manuscript (I marked some in the commented pdf) and should be avoided. **Fixed everywhere.**
29. P. 7, L. 17: “were repeated with 30 distinct male beetles for all combinations of contact mode” – This may be misunderstood as if all combinations were done with all 30 beetles. Please rephrase more clearly. **Rephrased: “Five force measurements were subsequently performed, each on a fresh spot of the substrate, and were averaged to avoid pseudo-replication during data analysis. Experiments were repeated with distinct male beetles for each combination of contact mode (“in air”, “underwater: bubble” and “underwater: no bubble”) and substrate chemistry (hydrophilic and hydrophobic), using 5 beetles for each combination. Thus, 30 distinct beetles were used in total” (p.3,ln.315-321)**
30. P. 9, L. 3: “its contact line at the water surface shifted” – I am not sure if I understand this statement entirely correct. Can you rephrase more clearly? **Rephrased: “Five force measurements were subsequently performed, each on a fresh spot of the substrate, and were averaged to avoid pseudo-replication during data analysis. Experiments were repeated with distinct male beetles for each combination of contact mode (“in air”, “underwater: bubble” and “underwater: no bubble”) and substrate chemistry (hydrophilic and hydrophobic), using 5 beetles for each combination. Thus, 30 distinct beetles were used in total” (p.3,ln.333-336)**
31. P. 14, L. 9-11: “We thus defined the size parameters, $-f = D_h = (2sf)$ and $-b = D_p = (2sb)$ for the fluid and bubble respectively, to conveniently scale their volumes relative to the hair and pad diameters they are pinned to.” – So if I understand correctly, if $\phi_f = 1$ then the liquid below single hairs has a volume that equals the volume of a sphere with a diameter

equal to the hair diameter? Can you express the physical meaning of these parameters in the text? Yes, that is correct. Larger values of ϕ_f (ϕ_b) represents a smaller volume of liquid (bubble) relative to the hair (pad) that it is pinned to. (p.5,ln.484-487)

32. P. 14, L. 22: “ F_{net} ” – What exactly is F_{net} ? Sum of surface tension and laplace pressure? No, it’s the net force of the array (p.5, eq. 1 and 2)
33. P. 16, L. 5-7: Please quantify what is meant with ‘high’ and ‘low’ adhesion here. Also, a description of general shape of the force curves would be more meaningful. Quantified as percentage of maximum force (p.6,ln.591-594). Force curve is also described in brief (p.6,ln.607-610)
34. P. 20, L. 7-8: “The contribution of the bubble to the net adhesion force is small regardless of its volume, when compared to the whole pad” – Please rephrase this statement in a quantitative manner. Also, it may be helpful to show the ratio of bubble force and pad force as a function of bubble volume in Figure 7. Bubble contribution has been quantified as less than 3% (p.7,ln.755). Since Figure 7 has been updated to show forces in μN units, we believe reporting the actual adhesion values predicted for the bubble can be more meaningful for comparison with experimental insect adhesion values.
35. P. 21, L. 13-14: “with a steep decrease (increase) in adhesion force on hydrophilic (hydrophobic) substrate as the volume decreases” – This could be rephrased more elegantly and clearly. Rephrased: “Five force measurements were subsequently performed, each on a fresh spot of the substrate, and were averaged to avoid pseudo-replication during data analysis. Experiments were repeated with distinct male beetles for each combination of contact mode (“in air”, “underwater: bubble” and “underwater: no bubble”) and substrate chemistry (hydrophilic and hydrophobic), using 5 beetles for each combination. Thus, 30 distinct beetles were used in total” (p.7,ln.766-776)
36. P. 23, L. 11: “If we choose $a=30 \text{ mN m}^{-1}$ and $w=40 \text{ mN m}^{-1}$ ” – From Table 1 I understand that you controlled ratios of the different interfacial tensions. Please rephrase accordingly. The updated results no longer uses constant interfacial tension ratios (see Table 1). Thus, the above confusion should be resolved.
37. P. 24, L. 3-4: “1. The resulting change in f_a and f_w will further decrease this number” – This statement is not clear to me. Rephrased: This discrepancy could be due to our assumptions of the oily fluid’s interfacial properties, which are not known for the ladybug beetle. Sensitivity analysis of the model does in fact show that the relative adhesion underwater when compared to that in air is sensitive to the fluid’s interfacial tension values in air and water (p.8,ln.867-872)
38. P. 24, L. 3-4: “In our experiments, however, not all hairs make a perfect contact with the substrate despite our best efforts to align the pad parallel to the surface.” – Albeit correct, this sentence illogically separates the foregoing and following sentences. I suggest shifting this sentence. It is not clear to us how the sentence position is illogical. (p.8,ln.881-884)

Figures

39. Figure 1: This figure nicely illustrates the experimental setup.

In the bottom right inset with the force/area-curves, the different phases were not clearly visible on some of my screens. I suggest using more distinct visual means to denote the different movement phases. **Color and pattern updated**

Also, why does the contact area curve not return to 0 after contact is lost?

This is due to the leftover residue of the secretion post contact which is being considered as 'area' by the image processing algorithm used (see also response to comment #7)

39. In the pause phase, I see that force and area reached nearly but not entirely constant values. Therefore, the text statement ("removed any viscoelastic effects before finally retracting the leg away from the substrate") should be slightly rephrased. **Reworded to "minimized any viscoelastic effects" (p.3,ln.296)**

40. Figure 2: I have several comments.

Especially for the low number of datapoints per boxplot (5), I suggest showing the underlying data points to provide all possible data. **Underlying points shown as dark markers.**

I find the appearance of modelling results in this figure surprising. The whole previous section was on experimental work, and now I see the results (and a long list of used parameters) of a so-far barely mentioned modelling efforts. Regarding the model parameter values: It would be more effective and comprehensive to shift this information to table 1, where further settings can be found. **Table 1 has been updated to show these values and the related text detailing the model parameters has been removed from the figure caption**

Also, the used symbols to indicate model results and outliers are confusing. At first glance I thought the crosses are outliers, and the diamonds are too small. I invite the authors to redesign this aspect of the figure more clearly.

Underlying data points are shown in black, modeling results are shown in coloured markers.

Finally, statistical differences between the different treatments could be indicated also in the figure.

This would make the figure look very busy, and hence we decided not to plot this data.

41. Figure 4:

If I understand correctly, the gap width d was left undetermined in the theoretical model, and was finally determined by picking the minimum of the force-distance-curve (this applies to all shown data, right?). This would mean that gap widths may vary between all the different model predictions, which raises several questions.

- 1) Was d always large enough so the used capillary adhesion model was applicable?
- 2) If d varied substantially between different computations and reached small values ($< ca. 10\text{ nm}$) in some cases, should variations in contributions of hydrodynamic or van der Waals forces also be taken into account (or at least discussed appropriately)?

With the updated Figure 5 showing distance in real units, the gap distance is of the order of 300-400 nm, which is large enough for capillary forces to be valid. This rules out van der Waals forces. Hydrodynamic forces could however exist, but are not considered for this study. Its contribution can however be expected to be similar for each contact mode presented here.

42. Figure 5:

I am wondering if this figure could be shifted to the supplement. In my opinion, the shown curves are not substantial for the main story, only the negative peak values (i.e. max adhesion) are. These values could easily be implemented in Figure 6.

We believe it is relevant to show the actual force distance curves of the array to illustrate the low contribution of the bubble throughout the distance range. It also clarifies the use of peak adhesion forces in Figures 7 and 8.

The titles of the figures (hydrophilic and hydrophobic) do not agree with above used ones (Glass and PFOTS). Please use consistent nomenclature throughout all figures.

Changed accordingly.

What is the horizontal grey dotted line? Zero force? If so, I suggest using a less prominent line style. Changed accordingly

43. Figure 6: The symbol used for surface tension does not agree with previously used nomenclature. Changed accordingly

For PFOTS-Underwater, it is not clear what 'higher adhesion' relates to; to glass or to a dry situation? Changed to "High adhesion"

44. Figure 7: Due to the normalisation I find interpretation of the applied bubble volume difficult. Which range of volumes is biologically meaningful? Please discuss accordingly, and, if necessary, limit the shown results to that meaningful range.

Figure 7 updated to real unit to simplify interpretation

45. Figure 8:

What is the unit on the x-axis? Micrometers? And is the whole range of Dh biologically meaningful?

What units do have Dp and Vb in the small box?

And why specifically mention symbol specifications for the 'underwater: bubble' case in the caption?

We updated Figure 8 and replaced the dimensionless units with real units in order to simplify interpretation. Furthermore, we kept the hair length fixed instead of using a fixed aspect ratio, focusing solely on the effect of changing hair diameter. The inset plot also clarifies the contact splitting effect.

Other, major

46. P. 19, L. 8-9: “The net force in the underwater: bubble case mainly depends on the proportion of hairs inside and outside the bubble (equation (2)). For the given bubble volume, only part of the hairs make contact with the surface inside the bubble for the hydrophilic case” – I could not find in the results any quantification of the fraction of hairs in and outside of the bubble. This would be an interesting additional result, for example in Figure 7!

Figure 7 now includes an inset plot showing the fraction of hairs inside and outside the array.

47. P. 5, L. 3: “male ladybug beetles (*Coccinella septempunctata*)” – In the introduction you mention that you chose this species because of the discoidal hair tip shape and superior attachment performance, also underwater. However, these animals to my knowledge are terrestrial and I am wondering about the relevance of good underwater attachment for this species. Can you elaborate?

Our study is a follow-up on the earlier study by Hosoda et. al. (2012, Proc Biol Sci 279(1745)), 4236–42.) where it was showcased that even a terrestrial insect can exhibit adhesion underwater. Although it is a rather artificial scenario (beetle being totally submerged underwater), we believe that an insect can encounter very wet or flooded surfaces and thus has to cope with adhesion under wet conditions. We also hope that our study encourages further research of underwater adhesion in other terrestrial animals especially in light of bio-mimicry.

Other, moderate

48. P. 3, L. 6: “Some aquatic insects like diving beetles or midge larva use suction cups to adhere to surfaces” – Further works on aquatic insect attachment that may be useful:

Ditsche, P. & Summers, A. P. Aquatic versus terrestrial attachment: Water makes a difference *Beilstein Journal of Nanotechnology*, Beilstein-Institut, 2014, 5, 2424-243; Ditsche-Kuru, P.; Barthlott, W. & Koop, J. H. At which surface roughness do claws cling? Investigations with larvae of the running water mayfly *Epeorus assimilis* (Heptageniidae, Ephemeroptera) *Zoology*, 2012, 115, 379-388. **References cited (p.1,ln.93)**

49. P. 15, L., 12-13: “forces were normalized w.r.t. γ ” – The axis labelling of several figures indicates normalisation by γ and other parameters. Please check throughout the manuscript for correct labelling.

Also, I am wondering about the use of this normalisation. Interpretation of the results, and comparison to the experimental data as well as to literature data would be much easier in a non-normalised format. **Figures are no longer normalized**

50. P. 23, L. 12-13: “Since the bubble’s volume is kept constant, it will then have a lesser space available to occupy between the pad and the substrate.” – How much does gap width d vary between cases relative to L ? Is this variation negligible? Please discuss accordingly.

Gap width, d , is negligible compared to hair length, L . Due to the small volume, the fluid thickness is at least 2 orders of magnitude smaller than hair length. Also, the above mentioned effect was a result of the fixed aspect ratio assumption. Figure 8 has been updated to follow fixed hair length assumption, and thus, the bubble’s shape should remain similar throughout, unlike before.

51. P. 25, L. 14-17: “For example, ants are known to possess smooth adhesive pads which secrete a fluid containing oily substances⁹. It has been reported that some ants show similar adhesion on hydrophobic substrates under wet and dry conditions²⁵, similar to what we see in a ladybug.” – Here you compare hairy and smooth pads. Is there even a bubble in smooth pads?

No, the comparison has been made by considering the non-bubble scenario, since the we did not find any reported literature showing bubble entrapment in smooth pads when underwater. We include this comment on ants to highlight that the wetting of the fluid underwater could also potentially explain its underwater adhesion. The paragraph has been reworded for clarity. (p.9,ln.927-935)

52. P. 2, L. 5: “that an air bubble is not a prerequisite for their underwater adhesion” – This is true on hydrophobic substrates. On hydrophilic ones, the bubble does help to prevent adhesion loss due to substrate wetting (see figure 5).

Our experiments however don’t show significantly high adhesion for the hydrophilic case when there is a bubble trapped. The model overestimates this scenario, probably because it doesn’t consider interfacial water layer and its drainage on glass which will influence contact and adhesion.

Other, minor

53. P. 7, L. 9: “(roughly 3 mm water level)” – How does this compare to the size of legs or body of the beetles? How deeply were the legs covered with water? Mentioning average animal body size would be helpful here.

The beetles are roughly 5 mm long. The legs were completely submerged underwater. (p.3,ln.304)

54. Figure 3: It would be helpful if you indicated all geometrical parameters in this figure. For example, single hair liquid volume V_f , hair diameter D_h , etc. Updated.

Supplement

55. P. 1, L. 13-14: “An appropriate geometry refinement routine was chosen” – Which method exactly was used? Please specify. Reworded (p.1,ln.13-14)

56. P. 4, L. 4: “should be negligible” – As in the main document, I am missing a quantitative statement on the bubble contribution to total adhesion. Based on such a statement, the reader can decide him-/herself if the bubble is negligible. **The preceding sentence mentions the actual values. Percentage contribution is also included explicitly now (p.8, ln.855).**
57. Videos 1 and 2 show that also the 2nd-distal tarsal pad makes contact with the substrate. This disagrees with a statement in the main text “For the test, we only allowed the distal pad to come into contact with the substrate thus minimising partial or bad contact of the other one.” How often did contact of two pads occur? Please elaborate, take into account in data analysis and discuss, if necessary.
- 6 out of the 30 experiments had the proximal pad making a partial contact at the edge. However, it should not have a significant contribution as the obtained adhesion values lie in the same range as the ones without the proximal pad contact.**