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.

   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 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.

   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?

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

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?

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?

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 um sliding, 10 um 2nd indentation, 1 s pause) were chosen based on some earlier published works or arbitrarily? Can you elaborate?

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.

    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.

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, 952-960*). 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?

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.

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?

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.

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.

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.

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.

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.

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.

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?

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?

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.

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

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.

    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.

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?

19. P. 19, L. 3-4: "Additionally, the interfacial tension of the oily fluid underwater (fw) is twice that of in air (fa)." – A literature reference would help to strengthen this statement.

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   Dh)," – 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.

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.

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.

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?

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?
    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.
    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?
    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.

    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 elsewise, the 'gecko excursion' needs to be embedded more smoothly in the manuscript.

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.
26. P. 3, L. 13: "[...] can attach quite well [...]" – Please quantitatively specify this statement.

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.

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.

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.

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?

31. P. 14, L. 9-11: "We thus defined the size parameters, -f = Dh=(2sf) and -b = Dp=(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?

32. P. 14, L. 22: "*Fnet*" – What exactly is *Fnet*? Sum of surface tension and laplace pressure?

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.

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.

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.

36. P. 23, L. 11: "If we choose a=30 mN m-1 and w=40 mN m-1" – From Table 1 I understand that you controlled ratios of the different interfacial tensions. Please rephrase accordingly.

37. P. 24, L. 3-4: "1. The resulting change in   fa and   fw will further decrease this number" – This statement is not clear to me.

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.

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.

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

In the pause phase, I see that force and area reached nearly but not entirely constant values. Therefore, the text statement ("removed <u>any</u> viscoelastic effects before finally retracting the leg away from the substrate") should be slightly rephrased.

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.

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.

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.

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

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 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)?

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.

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.

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

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

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

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.

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?

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!

47. P. 5, L. 3: "male ladybug beetles (*Coccinella septempuctata*)" – 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?

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.

49. P. 15, L., 12-13: "forces were normalized w.r.t. fasf" – 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.

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.

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?

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).

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.

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

Supplement

55. P. 1, L. 13-14: "An appropriate geometry refinement routine was chosen" – Which method exactly was used? Please specify.

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.

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.