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Title: Identifying risk factors for exposure to culturable allergenic moulds in energy efficient homes by using highly specific monoclonal antibodies

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Abstract: The aim of this study was to determine the accuracy of monoclonal antibodies (mAbs) in identifying culturable allergenic fungi present in visible mould growth in energy efficient homes, and to identify risk factors for exposure to these known allergenic fungi. Swabs were taken from fungal contaminated surfaces and culturable yeasts and moulds isolated by using mycological culture. Soluble antigens from cultures were tested by ELISA using mAbs specific to the culturable allergenic fungi Aspergillus and Penicillium spp., Ulocladium, Alternaria, and Epicoccum spp., Cladosporium spp., Fusarium spp., and Trichoderma spp. Diagnostic accuracies of the ELISA tests were determined by sequencing of the internally transcribed spacer 1 (ITS1)-5.8S-ITS2-encoding regions of recovered fungi following ELISA. There was 100% concordance between the two methods, with ELISAs providing genus-level identity and ITS sequencing providing species-level identities (210 out of 210 tested). Species of Aspergillus/Penicillium, Cladosporium, Ulocladium/Alternaria/Epicoccum, Fusarium and Trichoderma were detected in 82% of the samples. The presence of condensation was associated with an increased risk of surfaces being contaminated by Aspergillus/Penicillium spp. and Cladosporium spp., whereas moisture within the building fabric (water ingress/rising damp) was only associated with increased risk of Aspergillus/Penicillium spp. Property type and energy efficiency levels were found to moderate the risk of indoor surfaces becoming contaminated with Aspergillus/Penicillium and Cladosporium which in turn was modified by the presence of condensation, water ingress and rising damp, consistent with previous literature.

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18th October 2015

Dear Professor Domingo,

RE: ER-15-1318 – Revision of manuscript entitled "Identifying risk factors for exposure to culturable allergenic moulds in energy efficient homes by using highly specific monoclonal antibodies".

We would like to thank the editor and reviewers for their comments, which we received on 14th October 2015. Our responses to the reviewers' comments are shown in black italics below. Changes are referenced with the relevant pages and line numbers in the manuscript alongside the track changes.

We believe that the changes we have made to the manuscript address the comments made by the reviewers (please see text below and attached documents). We can also confirm that the responses from reviewer #5 had previously been addressed prior to our submission to Environmental Research. We have nevertheless studied these detailed comments again with a view to making further improvements. However, given that we had already made substantive refinements in response to this reviewer, we feel that additional correction is not warranted.

If you have any further specific questions on the paper please feel free to contact us. Many thanks for your kind consideration and we look forward to hearing of your final decision.

Yours sincerely,

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*Response to Reviewers

ER-15-1318 – Response of authors to reviews of manuscript entitled "Identifying risk factors for exposure to culturable allergenic moulds in energy efficient homes by using highly specific monoclonal antibodies".

Reviewer's comments:

Reviewer #4: This study used monoclonal antibodies (mAbs) to identify allergenic culturable fungi isolated from visible mold growths in energy efficient homes. The study found good agreements between mAbs and DNA sequence-based identification methods. I think the study has merits for the researchers in the areas of allergy, mycology, and indoor science. My comments on this paper are as follows:

Major comment

The study concludes that the use of culture and mAbs can be an alternative technique to the DNA sequence-based method. In the current version of the manuscript, however, I could not find the advantages of the mAbs method over the ITS sequence-based methods. What are the advantages of the mAbs method? The fungal ITS sequences are highly variable, allowing at least for the genus-rank identification (species-rank identification is mostly possible), while the mAbs method cannot differentiate some closely-related genera such as Aspergillus and Penicillium. The sequencing-based methods are also easy to perform. Please clarify the advantages of the mAbs method. It is OK if the method was used for scientific interests, but it is better to state the advantages if the method is intended for future risk assessment studies.

Author's response

Pages 20/21, lines 472 to 480; inserted the following text and reference Prattes et al., 2014

A major advantage of mAbs is their adaptability to field-based diagnostics such as lateral-flow assays (LFA). To this end, mAb JF5 has been used to develop a commercially available LFA for point-of-care diagnosis of invasive pulmonary aspergillosis in immunocompromised patients (Thornton, 2008; Prattes et al., 2014). Similar LFAs incorporating the other mAbs described here could be used as monitoring tools to track contamination by known allergenic fungi. The speed, low cost and simplicity of such assays compared to relatively expensive and sophisticated laboratory-based DNA identification methods could simplify the risk assessment process.

Specific comments

Page 2 Line 12
Please state 100% concordance out of how many samples compared.

Page 2, line 39; added
(210 out of 210 tested)

Page 4 Line Lines 19-22
Please include reference(s) that show culturable spores evoke greater inflammatory diseases than non-culturable spores.

Page 4, lines 94 and 95; references added
Lee et al., 2006 and Sercombe et al., 2004.

Page 9 Line 15
Ambient temperature seems ambiguous. I assume it is indoor temperature as the authors state these hygrothermal data were taken from each room. Please clarify whether they are indoor or outdoor temperature.

Page 9, line 202; added the following for clarification;
Indoor ambient air temperature (^{o}C), relative humidity (%), dew point temperature (^{o}C) and vapour pressure (kPa) readings were recorded from each room surveyed. *We also collected the same readings from directly outside each property.*

Page 9 Line 22
The definition of "T Diff" is ambiguous. Is it indoor dew point temperature minus wall surface temperature, or wall surface temperature minus indoor dew point temperature? Please clarify.

Page 9, line 209; added the following for clarification;
Risk of condensation was measured by the difference (T Diff ^{o}C), *which is the difference between ambient dew point temperature (±0.3^{o}C) and the external wall surface temperature (^{o}C) of each room surveyed. In accordance to the manufacturers meter settings and guidelines, we categorised* T Diff as ≤0°C =condensation, >0 to ≤3°C = risk of condensation and >3°C no risk of condensation.

Page 10 Lines 21-22
Please include not only percentage values, but also absolute numbers of the samples.

Page 10, line 234; amended;
Samples were collected from contaminated surfaces located in the bathroom, main bedroom, child's bedroom, the hall way, kitchen, landing, living room and utility, *making up 21.9% (n=46), 30.5% (n=64), 12.9% (n=27), 6.2% (n=13), 5.7% (n=12), 1% (n=2), 19.5% (n=41) and 2.4% (n=5) of the total samples, respectively.*

Page 11 Lines 2-5
Was stroking the swabs to the surface of MEA plates done at each sampling house, or in the laboratory? Please clarify.

Page 11, line 239; added;

Fungal sampling *within the home*

Surface swabs *were* taken using sterile cotton buds wetted with sterilised water. *For each of the rooms surveyed,* lawns of fungal debris were then prepared *on-site*.……

Page 18 Lines 4-5
The finding of the inverse relationship between RH and Cladosporium seems unexpected. Cladosporium is thought to be hygrophilous. Please discuss why the inverse relationship was observed.

Page 18, line 529; added;

Our findings may have been influenced by the sampling method and period because we found no association with moisture readings or relative humidity*. In contrast to existing knowledge, increased relative humidity reduced the risk of Cladosporium, which is an abundant outdoor fungus (Flannigan et al., 2011). This may be a result of sampling within warmer months, limited sample size, limitations of taking spot measurements that do not take into account of fluctuations and residents opening their windows prior to the survey, which means indoor conditions reflecting outdoor humidity levels (Appendix E).*

Reviewer #5:

In this paper presented the authors have used an interdisciplinary approach integrating asset management, mAb- and nucleic acid-based detection methods, and epidemiological techniques to investigate the relationship between household energy efficiency and risk of allergenic fungal exposure. Specifically, they determine whether 1) signs of dampness, condensation and fungal odour, 2) increased household energy efficiency and 3)
behavioural/housing characteristics, increase the risk of indoor surfaces being contaminated with these allergenic fungi. Especially the specific aims 2 and 3 are interesting and have not been studied much. The paper has been written well and the epidemiological part of the study with is quite extensive when considering the methods used (environmental measures and property data), even though the number of households are quite low. The main problem of the study is the exposure assessment of the study and the relevance of the findings because of the sampling and analyzing strategy used, which are discussed later in detailed comments

Author's response

We thank the reviewer for their comments and can confirm that the authors had previously addressed these comments prior to submitting the manuscript to Environmental Research.

Introduction

The authors might want to consider adding some references to the sentences on the lines 94-97.

Word "infiltration" could be replaced with "concentrations/levels/occurrence…" on the line 97.

It is stated: "use of less sophisticated but nevertheless highly accurate monoclonal antibodies (mAbs) that provide quick and cost-effective means of tracking fungi at the genus, species or even isolate level (Thornton et. al. , 2002)". This is a bit confusing, since the method presented here involved several steps: cultivation of the sample, gross identification of the fungi, isolation of the colonies, reculturing of the colonies and the monoclonal antibody assay. This does not sound quick and cost-effective. If the monoclonal antibody assay would have been applied directly to the samples taken from surfaces and would be presented in quantitative manner, it would have be easy to agree to this sentence.

Author's response

The manuscript had been amended to address the above comments, with an emphasis that the method adopted was qualitative and stressed the strengths and limitations of this approach. We highlight that further research is required with respect to using mAbs quantitatively.

The authors aim to determine associations between certain environmental factors and occurrence of selected allergenic fungi. One might ask that are these selected allergenic microbes the most important ones that make a difference when considering the health effects due to especially moisture and mold damage? The health effects associated with moisture damage are most likely due to also other mechanism than allergy. One must also consider that most of these selected microbes are very common in indoor and/or outdoor air, so it is quite probable that those will occur on the moist surfaces. The aim is clearly stated, but how much relevant new information is gained by fulfilling the aims? (this will be discussed also later)

Author's response;

We have clarified the originality of the study aims, which are reflected throughout the manuscript.

Methodology
Please, explain the participation a bit better, eg. how many of 83 customers agrees and participated later?
Please, explain how representative sample customers of social housing are when considering the population?
Please, clarify whether a customer represents a household?
The sentence on the line 215: "The Index of Multiple Deprivation (IMD) score has been shown to have a strong relation with health in both rural and urban areas (Jordan et. al. , 2004), and found to be associated with increased risk of fungal contamination (Sharpe et. al., 2015a)." could be used either in the introduction or in the discussion.

Please, explain, what were the criteria for fungal sampling? How were the suspected surfaces chosen? Were the sampled surfaces all the surfaces that had visible mold meaning that all the contaminated surfaces were sampled? If not, were all the houses sampled in the same manner taking as representative sample of contaminated surfaces. Was the sampling done parallel with the collection of environmental data? Please, include information, how many sites per home on average were sampled and which room. Please, clarify the sampling section. In addition, clarify the strategy for isolation of colonies. Were all the colonies isolated and further analyzed? If not, how was the selection done?

Author's response;

The methodology has been updated to reflect the above comments, and we have moved the statement "The Index of Multiple Deprivation (IMD) score has been shown to have a strong relation with health in both rural and urban areas (Jordan et. al. , 2004), and found to be associated with increased risk of fungal contamination (Sharpe et. al., 2015a)." to the strengths and limitations section;

Page 25, line 584; added

The Index of Multiple Deprivation (IMD) score was used because it has been shown to have a strong relation with health in both rural and urban areas (Jordan et al., 2004), and found to be associated with increased risk of fungal contamination (Sharpe et al., 2015a).

Please explain why results on Fusarium and Trichoderma were not included to the statistical analyses?
Please, clarify the relevance of using dichotomous variable: present/non-present? Was also the primary aim to show only the occurrence of selected microbes on the surfaces with visible mold growth? if yes, are authors suggesting that visibly moldy surfaces are more of a risk when containing at least one colony of these allergenic fungal species/groups? How about the area of the contaminated surface and the amount of microbial growth, are those of no interest? In addition, some of the environmental factors could have been built to at least with three categories: no, moderate and high or even continuous variable.
Please, explain how the factors were chosen for the adjustment of thestatistical models. Was the effect of multiple testing considered?

Results
"Fungi were recovered from main bedrooms (31.4%), bathrooms (20%), living rooms 354 (19.1%), children's bedrooms (12.7%) and the kitchens (6.4%)." Do the authors mean with the sentence that fungal samples were collected from these sites? If yes, this should be included to the section explaining the fungal sampling….most likely not, since the sum of percentages is not 100%. Or does this mean that fungal colonies were observed eg. in 6.4% of the samples collected from contaminated surfaces in the kitchens. Furthermore when reading the following sentences one might ask what essential knowledge is gained with the paragraph distribution on fungi explaining in which rooms these selected fungi are observed?

Discussion
The authors write: "Because of the intermittent nature of airborne spore production (Bush and Portnoy, 2001), we chose to swab contaminated surfaces directly and to use mycological culture for fungal isolation rather than spore traps." The authors should explain and justify their choice more detailed. How is exposure expected to happen? The main exposure route is typically considered be air. However, there is a huge spatial and temporal variation in airborne microbial concentrations and therefore short time air samples are hardly ever used for exposure assessment. . Instead, either eg. settled dust samples are used or microbial exposure is assessed by size of mold damage or moldy surface and/or composition of microbial growth on the damaged site.

I do not agree with the sentence "The use of mAbs to detect specific extracellular glycoprotein molecules in crude antigen samples prepared from mycological cultures allows simple
identification of different groups of allergenic fungi, removing the labour-intensive and, at times, ambiguous identification of fungi-based on visual characterisation of fungal propagules in air samples or taxonomic classification based on morphological characteristics in culture (Meheust et. al., 2013)." This method presented here does not allow simple identification: It includes culturing of a sample and incubation, gross identification of a colony, isolation of a colony, reculturing of a colony and incubation and monoclonal antibody assay. In microscopical identification, one needs to do the

culturing of the sample and later after incubation identification of a colony with microscope. The identification of especially the genera included to the statistical analyses (Cladosporium, Penicillium/Aspergillus and Alternaria/Ulocladium) and within those groups presented here is quite straight forward and easy. As stated earlier, the method would be robost and easy, if the assay would have applied directly to the surface sample and it would produce a result of genera or group specific concentration per cm2. In addition, the limitation of antibody based assay having differences between different lots is not discussed at all.
The comparison done against ergosterol and b-glucan feels irrelevant, since these methods are aiming only at quantitation of fungi, not for identification. At the same time, authors do not discuss at all that they are missing the quantitation totally (see next paragraph).
The authors write: "Using the mAb-based ELISAs, Aspergillus/Penicillium, Cladosporium,
Ulocladium/Alternaria/Epicoccum, Fusarium, and Trichoderma spp. were shown to constitute 82% of the fungal species recovered from contaminated surfaces, which may represent a respiratory health risk in susceptible individuals (Sharpe et. al., 2014a)." It is not clear, how many contaminated surfaces per home have been included, or how large the contaminated areas were and how much microbial growth was observed (cfu/cm2), which as such are important factors that may affect health. The authors seem to only consider the occurrence of selected allergenic fungi, which may be also relevant, but does not given any quantitative measure for the exposure.

In total 204 isolates were tested with ELISA. Were these all the colonies that were isolated from the contaminated surfaces? Do authors refer to half of 204 in the following sentence? "Nearly half of the fungal isolates were collected from bedrooms within the properties surveyed, where occupants spend the majority (~8 hrs) of their time indoors" It feels quite unlikely that authors would have found only 204 colonies out of 41 homes with most likely multiple contaminated surfaces. It would be important to clarify the strategies for sampling and also the isolation of colonies. The authors underline the importance of their results but do not convince the reader.
The authors present quite much data in the Tables 2 -6. The importance and relevance of these results are hardly discussed. As mentioned earlier, the authors should consider how important is the data based only on occurrence? What does this qualitative data add to the science? What are the mechanisms that may be behind the observed associations in these tables 2-6? Are the significant associations real, logical and expected findings ie. following the hypotheses because of which those have been studied? Please consider the multiple comparisons made?
The authors state: "Strengths of our study include an interdisciplinary approach that uses asset management, molecular and epidemiological techniques to investigate the relationship energy efficiency and risk of allergenic fungi." I can be agreed that the design and the epidemiological part of the study is the strength of the study, but at the same time the exposure assessment without quantitative measures and well defined sampling strategies are the weaknesses of the study. The identification of the selected fungal genera or groups seem to work, but as asked already several times, it is not obvious why the identification is needed to be done in a such way and why the authors did not do it quantitatively, so that the amount of fungal growth as concentration per area and area of the damaged surfaces would have included. The limitations of the study should also be discussed in the light of things discussed in this review.
The authors do not make any clear conclusions of their results and have no conclusions based on the actual aims presented in the introduction.

Author's response;

The manuscript had previously been amended to reflect these comments.

Highlights

- Monoclonal antibodies were used to track culturable allergenic moulds in homes
- Allergenic moulds were recovered from 82% of swabs from contaminated surfaces
- The mAbs were highly specific with 100% agreement to PCR of recovered fungi
- Improvements to energy efficiency lowered risk of exposure to allergenic fungi

*REVISED Manuscript (Marked-up with changes)
Click here to view linked References

Environmental Research

1
Identifying risk factors for exposure to culturable allergenic moulds in energy efficient homes by using highly specific monoclonal antibodies

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27 **Abstract**

28 The aim of this study was to determine the accuracy of monoclonal antibodies
29 (mAbs) in identifying culturable allergenic fungi present in visible mould growth in
30 energy efficient homes, and to identify risk factors for exposure to these known
31 allergenic fungi. Swabs were taken from fungal contaminated surfaces and culturable
32 yeasts and moulds isolated by using mycological culture. Soluble antigens from
33 cultures were tested by ELISA using mAbs specific to the culturable allergenic fungi
34 *Aspergillus* and *Penicillium* spp., *Ulocladium*, *Alternaria,* and *Epicoccum* spp.,
35 *Cladosporium* spp., *Fusarium* spp., and *Trichoderma* spp. Diagnostic accuracies of
36 the ELISA tests were determined by sequencing of the internally transcribed spacer 1
37 (ITS1)-5.8S-ITS2-encoding regions of recovered fungi following ELISA. There was
38 100% concordance between the two methods, with ELISAs providing genus-level
39 identity and ITS sequencing providing species-level identities (210 out of 210 tested).
40 Species of *Aspergillus/Penicillium, Cladosporium, Ulocladium/Alternaria/Epicoccum,*
41 *Fusarium* and *Trichoderma* were detected in 82% of the samples. The presence of
42 condensation was associated with an increased risk of surfaces being contaminated
43 by *Aspergillus/Penicillium* spp. and *Cladosporium* spp., whereas moisture within the
44 building fabric (water ingress/rising damp) was only associated with increased risk of
45 *Aspergillus/Penicillium* spp. Property type and energy efficiency levels were found to
46 moderate the risk of indoor surfaces becoming contaminated with
47 *Aspergillus/Penicillium* and *Cladosporium* which in turn was modified by the presence
48 of condensation, water ingress and rising damp, consistent with previous literature.

49 **Key words*:*** Allergenic fungi, asthma, monoclonal antibody, antigen, ELISA

50 **Ethical Approval**

51 Ethical approval for this cross sectional study was granted by the University of Exeter
52 Medical School, application number 13/02/013.

53 **Funding**

54 Richard Sharpe's PhD scholarship was funded by the European Social Fund
55 Convergence Programme for Cornwall and the Isles of Scilly, and was undertaken in
56 collaboration with Coastline Housing.

57 The European Centre for Environment and Human Health (part of the University of
58 Exeter Medical School) is part financed by the European Regional Development
59 Fund Programme 2007 to 2013 and European Social Fund Convergence Programme
60 for Cornwall and the Isles of Scilly.

61 **Abbreviations**

62 ELISA: Enzyme-Linked Immunosorbent Assay
63 IAQ: Indoor air quality
64 mAb: Monoclonal antibody
65 OR: Odds ratio
66 SAP: Standard assessment procedure
67 VOC: Volatile organic compound
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74 **Introduction**

75 Increased exposure to indoor damp and associated fungal contamination is a
76 worldwide public health concern because of its association with an increased risk of
77 allergic diseases (Fisk et al., 2007; Mendell, 2014; Quansah et al., 2012), now
78 present in around a third of the European population (Annesi-Maesano and Moreau,
79 2009). Fungal growth on surfaces in homes increases resident's exposures to
80 elevated concentrations of spores and hyphal fragments (Sharpe et al., 2014c),
81 which in turn is influenced by the type of material (Andersen et al., 2011), moisture
82 (Flannigan et al., 2011), indoor air velocity, and the types of fungi present (Mensah-
83 Attipoe et al., 2014b). There is limited research assessing how the interaction
84 between occupant behaviours and the built environment regulates the diversity of
85 allergenic fungi (Sharpe et al., 2014b). This is important to consider because different
86 genera of allergenic fungi are associated with the development (Reponen et al.,
87 2011) and exacerbation of asthma (Sharpe et al., 2014b), and a phenotype of severe
88 asthma in sensitised individuals (Denning et al., 2014; Denning et al., 2006). Despite
89 current knowledge of the involvement of fungal allergens in the pathophysiology of
90 allergic diseases, fungi as a prominent source of allergens are still largely
91 neglected(Crameri et al., 2013).

92 Culturability of fungal propagules has a profound effect on the production of
93 allergens, with culturable spores having a greater potential to evoke inflammatory
94 disease than dead ones when deposited in the respiratory tract (Lee et al., 2006;
95 Sercombe et al., 2004). Furthermore, increased allergen production during spore
96 germination has been demonstrated (Green et al., 2003; Lee et al., 2006; Mitakakis
97 et al., 2001; Sercombe et al., 2004). Consequently, methods of identification are
98 needed that extend beyond categorisation of fungal contamination by the presence of

99 dampness and visible fungal growth, to detection of culturable moulds known to
100 cause allergic reactions such as *Aspergillus* (Gravesen et al., 1999; Patterson and
101 Strek, 2010; Shen et al., 2007), *Penicillium* (Gravesen et al., 1999; Shen et al.,
102 2007), *Ulocladium* (Gravesen et al., 1999; Kaur et al., 2010), *Alternaria* (Breitenbach
103 and Simon-Nobbe, 2002), *Epicoccum* (Bisht et al., 2000), *Cladosporium* (Breitenbach
104 and Simon-Nobbe, 2002; Gravesen et al., 1999), *Trichoderma* (Lübeck et al., 2000),
105 and *Fusarium* species (Verma and Gangal, 1994). Identifying risk factors that
106 promote the growth of these allergenic fungi can inform housing interventions aimed
107 at ameliorating disease symptoms in susceptible populations. Tailored housing
108 improvements offer a cost-effective approach to delivering healthcare to individuals
109 suffering from moderate to severe asthma (Edwards et al., 2011) and improving lung
110 function of individuals residing in, for example, mould contaminated water-damaged
111 homes (Norbäck et al., 2011).

112 The Environmental Relative Moldiness Index (ERMI), which encompasses a
113 range of fungal indicator species (Vesper et al., 2007) has been adopted, albeit
114 principally in the US, as a method for categorising the extent of indoor fungal
115 contamination. The index has been used to determine levels of risk to fungal
116 exposure in the home and to predict the occurrence of illness in homes (Vesper et
117 al., 2006). Based on mould-specific quantitative PCR (MSQPCR), it determines loads
118 of fungal DNA in dust samples and is being increasingly used because of its low
119 detection limit and high specificity (Méheust et al., 2013). While MSQPCR is precise,
120 it is based on nucleic acid-based detection methods that are unable to differentiate
121 between DNA derived from live and dead propagules. Furthermore, the US
122 Environment Protection Agency has not validated or peer reviewed MSQPCR or

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123 ERMI for public use, considering it to be a research tool only, despite firms offering
124 remediation services based on results of ERMI surveys.

125 No studies have investigated the combined use of culture and well-
126 characterised fungal-specific monoclonal antibodies (mAbs) as a means of detecting
127 and identifying culturable allergenic fungi indoors, or to use this approach to
128 determine potential risk factors that regulate their occurrence in homes. In this study,
129 we combine asset management, epidemiology, detection using mAb-based ELISA
130 and validation using Internal Transcribed Spacer (ITS) sequencing of fungi, to
131 determine potential risk factors that promote the growth of culturable allergenic
132 *Aspergillus*, *Penicillium*, *Ulocladium*, *Alternaria*, *Epicoccum*, *Cladosporium*,
133 *Trichoderma*, and *Fusarium* spp. in energy efficient homes. This is the first time, to
134 our knowledge, that mAbs have been used to assess how demographic and
135 environmental factors modify the growth of these allergenic moulds.
136

137 **Methodology**

138 **Study population**

139 Ethical approval for this cross sectional study was granted by the University of Exeter
140 Medical School, application number 13/02/013. The Cornish Health project was
141 conducted during 2013 and 2014 in collaboration with a social housing association
142 located in the SW of Cornwall, England which manages around 4,000 social housing
143 properties (Sharpe et al., 2015b). We worked closely with the social housing
144 associations customer services contact centre to recruit study participants from the
145 target population (customers of the social housing association) (Sharpe et al.,
146 2015b). Using a standard template (Appendix A), customers from 83 social housing
147 properties (those who contacted customer services between April and September
148 2013) were randomly selected and asked whether they wished to participate in the
149 Cornish Health project. Interested participants were subsequently sent a covering
150 letter and information sheets, and were then contacted by telephone five days after
151 the postage date of each letter to arrange a home visit. Written consent was obtained
152 using a form containing a series of scripted questions concerning participant
153 involvement in various elements of the study. We used face-to-face questionnaires to
154 collect demographic, behavioural and health data from participating adults (Appendix
155 B), which was followed by an environmental survey using a standardised template
156 (Appendix C).

157 **Property data**

158 Property records from the social housing association were obtained from the asset
159 management and stock condition database in February 2014 and merged using a
160 unique household identifier. Data included residency period, property age and build
161 type, type of heating, glazing, insulation levels, energy efficiency ratings and date of

any property upgrades. Energy efficiency ratings were calculated according to the Government's Standard Assessment Procedure (SAP). SAP 2009 was used for compliance with building regulations in England & Wales (BRE, 2013) for new builds (Part L1A) and existing buildings (Part L1B). It is the chosen methodology for delivering the EU performance of building directive (EPBD) and is used in the calculation and creation of Energy Performance Certificates (Kelly et al., 2012). SAP is calculated for both new and existing builds, and ranges from 0 to 120 with 120 representing the highest energy efficiency rating. SAP ratings were provided by the social housing provider and were auto-assessed using RDsap 9.91 (BRE, 2014) and taken from new build energy assessments (Department of Energy & Climate Change, 2014).

Socio-economic status (SES)

~~The Index of Multiple Deprivation (IMD) score has been shown to have a strong relation with health in both rural and urban areas (Jordan et al., 2004), and found to be associated with increased risk of fungal contamination (Sharpe et al., 2015a). For this reason w~~We obtained the IMD scores for 32,482 LSOAs (Large Super Output Areas) in England and Wales: each area contain a mean population of between 1,000 and 1,500 people (ONS, 2014). The score uses the English Indices of Deprivation 2010 to identify areas of England experiencing multiple aspects of deprivation, and were merged with our data using property full postcodes.

Questionnaire data

Questionnaires were designed to collect data on participant demographics on all occupants and environmental exposures thought to influence the risk of asthma initiation and/or exacerbation (Dales et al., 2008; Gaffin and Phipatanakul, 2009). Boxes were provided for either partner in the household to provide answers

187 (Appendix B). Questions covered participant age, sex, height, weight; smoking
188 status; employment; cleaning regimes; number of rooms carpeted; pets; health data
189 on asthma, allergy and chronic bronchitis or emphysema; heating / ventilation
190 regimes and whether participants thought damp/mould impacted their family's health.
191 We modified the LARES project questionnaire (Ormandy, 2009) and ISAACs
192 definitions (Asher et al., 1995) to assess the exacerbation of wheeze, and then
193 current asthma by asking participants if they had seen a doctor in the last 12 months
194 and/or take medication for asthma.

195 **Environmental data**

196 Home surveys were conducted throughout the year with 10, 5, 3, 10, 2, 2, 2 and 7
197 visits being carried out during April, May, June, July, May, September, December
198 and January 2013/14, respectively. A trained investigator (RS) carried out
199 environmental surveys using a Protimeter MMS2 damp meter Model: BLD8800
200 (General Electric, MA, US), which was calibrated according to the manufacturer's
201 settings. Visual inspections were made to identify areas of condensation, water
202 leakages and rising damp. Indoor aAmbient air temperature (°C), relative humidity
203 (%), dew point temperature (°C) and vapour pressure (kPa) readings were recorded
204 from each room surveyed. We also collected the same readings from and directly
205 outside each property. High moisture generating properties were assessed by
206 calculating excess vapour pressure (indoor minus outdoor vapour pressure), where a
207 limit of 0.6 kPa was set in accordance to the British Standard BS 5250:2011 (BSI,
208 2011). The following measurements and limits were set in accordance to the
209 protimeter manufacturer guidelines. Risk of condensation was measured by the
210 difference (T Diff °C), which is the difference between ambient dew point temperature
211 (±0.3°C) and the external wall surface temperature (°C) of each room surveyed. In

accordance to the manufacturer's meter settings and guidelines, we ~~readings and~~ categorised ~~as~~ T Diff as ≤0°C =condensation, >0 to ≤3°C = risk of condensation and >3°C no risk of condensation. Wall dampness was assessed using a non-invasive probe measuring relative moisture at 15 mm (two readings taken at 1m intervals from the top of the skirting boards), which ranged from 60 (dry) to high moisture content (999) relative scale. Wall dampness was categorised as <170 = dry wall, ≥170 but <200 = risk of damp and ≥ 200 = dampness. Relative humidity (≥65%), vapour pressure (>1 kPa), wall surface temperature difference of <3^{0}C (TDiff) and visual signs of dampness were used as dichotomous exposure variables.

Fungal sample plan

In parallel to the collection of environmental data, contaminated surfaces with visible fungal growth were identified and selected for sampling via a home walk through with each participant and from the environmental survey. We planned to take a single sample from each surface with visible fungal growth in a home (i.e. all individual contaminated surfaces were sampled). When there was more than one surface with visible fungal growth in a room or hallway, we extracted a single sample from each contaminated site. These locations were along the window recess, along the ceiling / wall junction and floor / wall junctions and in isolated locations with water damage (leaks or rising damp). We obtained a single sample from each surface (i.e. a wall, ceiling or floor area) in severe cases where whole surfaces had signs of condensation and visible fungal growth. Samples were collected from contaminated surfaces located in the bathroom, main bedroom, child's bedroom, the hall way, kitchen, landing, living room and utility, making up 21.9% (n=46), 30.5% (n=64), 12.9% (n=27), 6.2% (n=13), 5.7% (n=12), 1% (n=2), 19.5% (n=41) and 2.4% (n=5)~~16.6%, 33.1%, 10.4%, 2.5%, 9.2%, 1.2%, 21.5% and 2.5%~~ of the total samples,

237 respectively. Details of the number of samples taken from each property surveyed,
238 and within each room are provided in Appendix E. The location, clustering and
239 number of samples taken from each room and home are accounted for in our
240 statistical analysis plan described below.

241

242 **Fungal sampling within the home**

243 Surface swabs were taken using sterile cotton buds wetted with sterilised water.
244 Within For each of the rooms surveyed, lLawns of fungal debris were then prepared
245 on-site by gently stroking the swabs across the surface of malt extract agar (MEA)
246 culture plates containing the broad-spectrum antibiotic rifampicin (MP Biomedicals).
247 After transportation to the laboratory (within 48 hours), the plates were incubated
248 under a 16 h fluorescent light regime at 26°C. Fungi were separated on the basis of
249 gross morphological characteristics and sub-cultured on MEA as axenic cultures.

250 **Growth of fungi and preparation of antigens**

251 For antibody specificity tests, fungi were grown as two replicate MEA slope cultures
252 and surface washings containing soluble antigens were prepared using phosphate
253 buffered saline (PBS: 0.8% NaCl; 0.02% KCl; 0.115% Na_2HPO_4; 0.02% KH_2PO_4;
254 pH7.2) as described in Thornton (2001). Protein concentrations, determined
255 spectrophotometrically at 280 nm (Nanodrop, Agilent Technologies Limited,
256 Berkshire, UK), were adjusted to 60 µg ml^{-1} buffer. Fifty-µl volumes were then used to
257 coat the wells of Maxisorp microtitre plates (Nunc DIS-971; Thermo Fisher Scientific,
258 Leicestershire). After incubating overnight at 4°C, wells were washed four times with
259 PBST (PBS containing Tween-20, 0.05% (v/v)) and once each with PBS and dH_2O

260 and air-dried at 23°C in a laminar flow hood. The plates were stored in sealed plastic
261 bags at 4°C prior to ELISA tests.

262 **Enzyme-Linked Immunosorbent Assay**

263 Wells containing immobilised antigens were blocked for 10 min with PBS containing
264 1% (w/v) bovine serum albumin (A-2153; Sigma Chemical Company, Poole, United
265 Kingdom) and then incubated for 1 h with hybridoma tissue culture supernatants
266 containing fungus-specific monoclonal antibodies (mAbs). The mAbs used were mAb
267 JF5 (mouse IgG3 specific to *Aspergillus* and *Penicillium* spp. (Thornton, 2008)), mAb
268 ED7 (mouse IgM specific to *Fusarium* spp. Thornton and Wills, 2015), mAb MF2
269 (mouse IgM specific to *Trichoderma* spp. (Thornton et al., 2002)), mAb OX-CH1
270 (mouse IgM specific to *Cladosporium* spp. (Karpovich-Tate et al., 1998)), and mAb
271 PC3 (rat IgG2a specific to *Ulocladium*, *Alternaria*, and *Epicoccum* spp. (Karpovich-
272 Tate and Dewey, 2001)). After washing four times (5 min each time) with PBST, wells
273 were incubated with goat anti-mouse polyvalent (immunoglobulin classes IgG, IgA,
274 and IgM) peroxidase conjugate (A-0412; Sigma)(mAbs JF5, ED7, MF2, OX-CH1) or
275 goat anti-rat peroxidase conjugate (G-8154; Sigma)(mAb PC3), both diluted 1 in
276 1000 in PBST containing 0.5% (w/v) BSA, for a further hour. Wells were rinsed four
277 times with PBST, once with PBS and bound antibody was visualised by incubating
278 wells with tetramethyl benzidine (T-2885; Sigma) substrate solution (Thornton, 2001)
279 for 30 min. The reactions were stopped by the addition of 3M H_2SO_4. Absorbance
280 values were determined at 450 nm with an automated microplate reader (Dynex
281 Technologies, Billingshurst, UK). Working volumes were 50 µl per well, control wells
282 were incubated with tissue culture medium (TCM) containing 10% (v/v) foetal bovine
283 serum, and incubation steps were performed at 23°C in sealed plastic bags.

284 **Identification of fungi by analysis of the ITS regions of the rRNA-encoding gene**
285 **unit**

286 Representative isolates of antibody-reactive and antibody non-reactive fungi
287 recovered from the 32 households were used to determine the accuracy of the mAb-
288 based ELISA tests. Species were identified by sequencing of the ITS1-5.8S-ITS2
289 region of the rRNA-encoding gene unit (White et al., 1990) according to procedures
290 described elsewhere (Thornton et al., 2002) using the primers ITS1ext (5'-
291 GTAACAAGGTTTCCGTAGGTG-3') and ITS4ext
292 (5'TTCTTTTCCTCCGCTTATTGATATGC-3'). Newly determined sequences were
293 submitted to GenBank, and accession numbers KP794062 to KP794197 were
294 obtained. Species designations of recovered fungi are shown in Appendix D.

295 **Statistical analysis**

296 *Aspergillus*/*Penicillium*, *Ulocladium*/*Alternaria*/*Epicoccum*, and *Cladosporium*
297 groupings identified using the mAbs were used as our dichotomous outcome
298 variables for statistical analysis because we were interested in factors regulating their
299 presence or absence on contaminated surfaces. Due to a small sample size,
300 *Fusarium* and *Trichoderma* were omitted from these analyses. Our small sample size
301 also prevented us from developing multiple environmental categories or continuous
302 variables such as variations in temperature, humidity and vapour pressure.
303 Behavioural, built environment and survey data previously described as dichotomous
304 exposure variables were also used in our analyses. Descriptive statistics were used
305 to depict the demographic and housing characteristics of participating homes. In
306 order to help reduce the impact of multiple testing, we developed a detailed analysis
307 plan to investigate the demographic and built environment risk factors. In our
308 accordance to our analysis plan Pearson's chi-squared tests were used to assess

309 differences between our exposure and outcome variables of interest. We adopted a
310 survey methodology that resulted in multiple samples being taken from each house
311 with visible fungal growth. For this reason we used multilevel mixed-effects logistic
312 regression (fixing each household surveyed and the location of each sample) to
313 account for residual variance that may occur between groupings of fungal samples,
314 which was carried out using the meqrlogit command in Stata version 13.0 (Stata
315 Corp., College Station, US). We used an *a priori* in our adjusted models, which
316 included occupancy rates, outdoor ambient air temperature and whether households
317 said they ventilated to minimise dampness and fungal growth because these have
318 been found to modify the risk of fungal growth. Both chi-squared tests and logistic
319 regression was used to assess whether 1) signs of dampness, condensation and
320 fungal odour, 2) increased household energy efficiency and 3) behavioural/housing
321 characteristics, increased the risk of indoor surfaces being contaminated with
322 *Aspergillus*/*Penicillium*, *Ulocladium*/*Alternaria*/*Epicoccum*, or *Cladosporium* spp.

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333 **Results**

334

335 **Study characteristics**

336 The study achieved a response rate of 49% and the results are based on 41
337 participating households and 93 inhabitants. Children had a slightly higher
338 prevalence of doctor diagnosed asthma (30%) and allergy (18%) when compared to
339 adults (24% and 16%, respectively). A high proportion of homes had visible fungal
340 growth (78%) and the presence of a fungal odour (37%). Participant behaviours and
341 building characteristics varied (Table 1). Mean air temperature, relative humidity, dew
342 point temperature and vapour pressure readings taken from living rooms and main
343 bedrooms were slightly greater than ambient levels taken from outside each property.
344 Sixty per cent of rooms had windows open, and mean external wall surface
345 temperatures were greater than dew point temperatures, although homes had
346 varying levels of dampness related problems and the majority of homes had their
347 chimneys sealed with air vents (Table 2). The demographics of our target population
348 (those living in homes owned and managed by the social housing provider) differed
349 slightly from our study participants (Table 1) in terms of a mean age (36 versus 59
350 years), proportion of male residents (44 versus 41%) and occupancy rates (2.3
351 versus 1.7 persons per house). Household demographics of target homes were
352 similar to participating homes in terms of their IMD score (mean 28.4 versus 34.1),
353 build age (1967 versus 1968), energy efficiency (SAP 66.0 versus 65.7) and use of
354 gas heating (53 versus 55%) (Sharpe et al., 2015b).

355

356 **Use of monoclonal antibodies to identify fungi and confirmation of antibody**
357 **specificities by using ITS sequencing**

358 Five specific mAbs were used to identify fungal genera in ELISA tests of crude
359 antigen extracts from axenic fungal cultures. All five of the mAbs were shown to be
360 specific for their target species (Appendix D). Of the 204 fungal isolates tested by
361 ELISA (i.e. all fungal isolates taken from the original surface swabs of visible fungal
362 growth in participating homes), 40% reacted with mAb JF5 (specific for *Aspergillus*
363 and *Penicillium* spp.), 27% reacted with mAb CF1 (specific for *Cladosporium* spp.),
364 9% reacted with mAb PC3 (specific for *Ulocladium*, *Alternaria*, *and Epicoccum* spp.),
365 3% reacted with mAb ED7 (specific for *Fusarium* spp.), and 3% reacted with mAb
366 MF2 (specific for *Hypocrea* (*Trichoderma*) spp.). The remaining fungi were unrelated
367 species non-reactive with the five mAbs tested. ITS sequencing of 136 of the fungal
368 strains confirmed antibody specificities, with mAb JF5 reacting specifically with
369 strains of *Aspergillus candidus*, *A. flavus*, *A. oryzae*, *A. tennesseensis*, *A. tubigensis*,
370 *A. versicolor*, and strains of *Penicillium brevicompactum*, *P. chrysogenum*, *P.*
371 *concentricum*, *P. commune*, *P. copticola*, *P. corylophilum*, *P. crustosum*, *P.*
372 *expansum*, *P. glabrum*, *P. polonicum*, *P. toxicarium*, and *P. tricola*. Fungi reactive
373 with mAb CF1 were identified as *Cladosporium cladosporioides*, *C. lignicola, C.*
374 *ossifragi*, and *C. sphaerospermum*. Fungi reactive with mAb ED7 were identified as
375 *Fusarium oxysporum* and *F. solani*, while fungi reactive with mAb MF2 were
376 identified as teleomorphic or anamorphic *Trichoderma* spp. including *Hypocrea*
377 *atroviridis* (anamorph *T. atroviride*), *H. lixii* (anamorph *T. harzianum*), *H. viridescens*
378 (anamorph *T. viridescens*) and *T. viride*. Fungi reactive with mAb PC3 included the
379 target species *Alternaria alternata*, *Epicoccum nigrum*, and *Ulocladium obovoideum*,
380 and *Pyrenochaeta unguis-hominis*, a dematiaceous fungus belonging to the

381 mitosporic Pleosporaceae group that includes the genera *Alternaria* and *Ulocladium*
382 (Liu, 2011). The antibody also cross-reacted with a single isolate of the unrelated
383 allergenic fungus *Phoma herbarum*. None of the mAbs cross-reacted with the
384 unrelated fungi identified using ITS sequencing including the moulds *Acremonium*
385 *sclerotigenum*, *Bjerkandera adusta*, *Eutypa lata*, *Nectria maurtitiicola*, *Peniophora*
386 *lycii*, *Periconia byssoides*, *Pseudeurotium bakeri*, the yeasts *Candida famata*
387 (*Debaryomyces hansenii*), *Candida intermedia*, *Candida parapsilosis*, *Candida*
388 *spencermartinsiae*, *Cryptococcus diffluens*, *Meyerozyma guilliermondii*, *Rhodotorula*
389 *mucilaginosa*, or the yeast-like fungus *Aureobasidium pullulans*.

390 **Indoor condensation and presence of an odour**

391 We assessed whether the presence of condensation and odour were risk factors for
392 the target genera of interest. Signs of condensation increased the risk of surfaces
393 being contaminated with *Aspergillus*/*Penicillium* (OR 2.37; 95% CI 1.05-5.36) and
394 *Cladosporium* (OR 4.32; 95% CI 1.23-15.20) in the adjusted models, but not
395 *Ulocladium*/*Alternaria*/*Epicoccum* (Table 3). On further investigation the presence of
396 condensation increased the risk of all three groups of fungus when we combined
397 them into unadjusted models (OR 2.85; 95% CI 1.15-7.10) and adjusted models (OR
398 2.52 95% CI 1.06-5.99). Only *Aspergillus*/*Penicillium* was associated with signs of
399 water damage (plumbing leakages) and rising damp in properties surveyed (OR 2.08;
400 95% CI 1.02-4.23). The presence of a fungal odour was associated with increased
401 risk of *Cladosporium* in the unadjusted model (OR 2.96; 95% CI 1.19-7.32), but not in
402 the adjusted model. No association was observed with odour and
403 *Aspergillus*/*Penicillium* and *Ulocladium*/*Alternaria*/*Epicoccum.* We observed no
404 association between mean wall moisture readings (two taken at 1 m intervals to

405 assess water ingress in the building fabric) or other dampness measures (wall
406 surface temperature and vapour pressure) and risk of either fungus.

407 *Aspergillus*/*Penicillium* and *Ulocladium*/*Alternaria*/*Epicoccum* were not
408 associated with elevated relative humidity, but high relative humidity was inversely
409 associated with risk of *Cladosporium* (OR 0.40; 95% CI 0.17-0.91). On further
410 investigation (Appendix E) we found that mean relative humidity was similar in
411 ventilated and unventilated homes, we observed higher vapour pressure readings
412 (indoor minus outdoor readings) in unventilated homes. However, mean values did
413 not exceed those typically found in a UK property (0.5 to 0.6 kPa) during the winter
414 months (BSI, 2011).

415 **Energy efficiency and risk of allergenic fungi**

416 Increased household energy efficiency was inversely associated with
417 *Aspergillus*/*Penicillium* contamination in unadjusted models when SAP was between
418 ≥61-69 (OR 0.43; 95% CI 0.19-0.96), but not when SAP ≥69-81 (OR 0.64; 95% CI
419 0.31-1.30). Increased energy efficiency was inversely associated with risk of
420 *Cladosporium* contamination when SAP ≥66-69 (OR 0.39; 95% CI 0.20-0.76) and
421 ≥69-81 (OR 0.38 95% CI 0.16-0.87) in adjusted models. There was suggestive
422 evidence that high SAP was associated with increased risk of
423 *Ulocladium*/*Alternaria*/*Epicoccum* contamination (P=0.04) (Table 4). In additional
424 analyses, signs of condensation was associated with an increased risk of surfaces
425 being contaminated by *Cladosporium* contamination in homes with a SAP ≥66
426 (P<0.05), but not in low energy efficient homes. We also observed that properties
427 with a water leak or rising damp problems were associated with increased risk of
428 *Aspergillus/Penicillium* and *Cladosporium* in homes with a SAP ≥66 (P<0.05), but not
429 in low energy efficient homes. We observed no association with either model

430 between water leaks or rising damp and *Ulocladium*/*Alternaria*/*Epicoccum*
431 contamination.

432 **Demographic and housing characteristics and risk of fungus**

433 We assessed behavioural and built environment risk factors and the presence of
434 *Aspergillus*/*Penicillium* and *Cladosporium,* but excluded
435 *Ulocladium*/*Alternaria*/*Epicoccum* due to the lack of power in the analyses.
436 Participant homes that had been vacuumed prior to the home survey was inversely
437 associated with *Aspergillus*/*Penicillium* (OR 0.46; 95% CI 0.21-0.98). Only building
438 architecture appeared to modify the risk of fungus contaminating indoor surfaces,
439 with a reduced (OR 0.42; 95% CI0.22-0.81) and increased (OR 3.56; 95% CI 1.41-
440 9.05) risk of surfaces being contaminated by *Aspergillus*/*Penicillium* and
441 *Cladosporium* spp., respectively (Tables 5 & 6).
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449 **Discussion**

450 This is the first time to our knowledge that highly specific monoclonal antibodies
451 (mAbs) have been used to track culturable allergenic fungi contaminating the indoor
452 surfaces of social housing in the UK. Because of the intermittent nature of airborne
453 spore production (Bush and Portnoy, 2001), we chose to swab contaminated
454 surfaces directly and to use mycological culture for fungal isolation. By combining
455 mycological culture with highly specific mAbs we were able to detect specific groups
456 of fungi known to be involved in human allergic diseases (Sharpe et al., 2014a).
457 While the mAb-based ELISA method is qualitative, detecting fungal presence rather
458 than amount, its specificity (confirmed by ITS sequencing of recovered fungi) and
459 combination with culture allows identification of culturable allergenic fungi in contrast
460 to current MSQPCR DNA detection methods that are unable to differentiate between
461 live and dead fungal biomass. The use of mAbs that detect signature antigens unique
462 to these fungi removes the need for skilled identification based on morphological
463 characteristics (Méheust et al., 2013). While the mAbs used in this study were unable
464 to identify the fungi to species level, their accuracy in detecting individual genera
465 (*Cladosporium*, *Fusarium* and *Trichoderma*) or genus-level groupings
466 (*Aspergillus*/*Penicillium* and *Ulocladium*/*Alternaria*/*Epicoccum*), meant that culturable
467 allergenic fungi known to be involved in human respiratory diseases (Denning et al.,
468 2014; Simon-Nobbe et al., 2008; Thornton C.R and Wills O.E, 2015) could be
469 detected with a high degree of accuracy by using crude antigen extracts. Indeed, four
470 of the mAbs displayed 100% specificity for their target species, while mAb PC3
471 targeting *Alternaria*, *Epicoccum*, and *Ulocladium* cross-reacted with only a single
472 strain of *Phoma*, a genus not previously tested with this mAb [43]. A major advantage
473 of mAbs is their adaptability to field-based diagnostics such as lateral-flow assays

(LFA). To this end, mAb JF5 has been used to develop a commercially available LFA for point-of-care diagnosis of invasive pulmonary aspergillosis in immunocompromised patients (Thornton, 2008; Prattes et al., 2014). Similar LFAs incorporating the other mAbs described here could be used as monitoring tools to track contamination by known allergenic fungi. The speed, low cost and simplicity of such assays compared to relatively expensive and sophisticated laboratory-based DNA identification methods could simplify the risk assessment process.

The ELISAs represent a significant improvement on diagnostic procedures based on pan-fungal cell wall constituents such as ergosterol and β(1→3)-glucan that are unable to discriminate between fungi and which can be affected by non-fungal sources (Bush and Portnoy, 2001). Other immunoassays that have been used to monitor fungal exposure are based on allergen detection in airborne or settled dust samples. While these are able to detect single allergenic proteins they can, in some instances, be too specific, being unable to detect the presence of fungi that do not produce the precise allergens that the assays are designed to detect (Bush and Portnoy, 2001). Our method is not allergen-specific (i.e. not all of the species detected using the mAbs are allergenic). Consequently, immunoassays such as those described here that identify fungi directly using signature antigens, and those that detect the fungi indirectly using allergenic proteins, could be combined to provide a more accurate measure of exposure to allergenic species in the indoor environment. ~~While the method we adopted was qualitative and laboratory based, this technique combined with additional research, offers an opportunity to develop qualitative and quantitative approaches for identifying allergic fungi on-site via a diagnosis kit, which overcomes the limitations of lab based approaches.~~

498 Using the mAb-based ELISAs, *Aspergillus*/*Penicillium*, *Cladosporium*,
499 *Ulocladium*/*Alternaria*/*Epicoccum*, *Fusarium*, and *Trichoderma* spp. were shown to
500 constitute 82% of the fungal species recovered from contaminated surfaces with
501 areas of visible fungal growth. While the presence of these fungi in the home may
502 represent a respiratory health risk in susceptible individuals (Sharpe et al., 2014b),
503 further research is required to assess and compare exposures to elevated
504 concentration of these fungi, as well as the impact of other fungi not assessed in this
505 study. Nearly half of the fungal isolates were collected from bedrooms within the
506 properties surveyed, where occupants spend the majority (~8 hours) of their time.
507 *Aspergillus*/*Penicillium* and *Cladosporium* were the most frequent fungi identified,
508 which corresponds to other studies (Flannigan et al., 2011). Flannigan et al. (2011)
509 reported that spores of *Aspergillus*/*Penicillium* and *Cladosporium* were the most
510 abundant types in indoor air in US homes, making up 19.8% and 38.8% of the total
511 aerospora respectively, with *Aspergillus*/*Penicillium* exceeding outdoor
512 concentrations. These fungi can dominate indoor environments, where they have
513 been cultured from sputum of asthmatic and non-asthmatic individuals, and
514 implicated in the initiation or exacerbation of asthma (Agbetile et al., 2012). The
515 identification and control of these high risk fungi may help to improve health and
516 benefit asthmatic individuals (Meng et al., 2012), although the complex interaction
517 between occupant behaviours and the built environment must also be considered
518 (Figure 1).

519 In keeping with our findings, other studies have found that fungal diversity
520 (number and type of fungal genera) is modified by factors such as occupancy
521 patterns (Howden-Chapman et al., 2005), opening windows or use of extractor fans
522 (Dharmage et al., 1999; Zock et al., 2002), property type, age, architecture and,

523 presence of carpeting / vacuuming, build age and type (Chew et al., 2003; Dharmage
524 et al., 1999; Fairs et al., 2010; Reponen et al., 2013; Zock et al., 2002), type of
525 dampness problem (Gent et al., 2002; Zock et al., 2002), sun exposure (Howden-
526 Chapman et al., 2005), type of heating / ventilation (Dharmage et al., 1999; Fairs et
527 al., 2010) and the extent of household insulation (Semple et al., 2012). Reduced risk
528 of cold bridging (BSI, 2011) explains why increased household energy efficiency
529 reduced the risk of surfaces being contaminated by *Aspergillus*/*Penicillium* and
530 *Cladosporium* spp. Energy efficiency interventions have been found to lower the risk
531 of visible fungal growth (Sharpe et al., 2015b), although this is reliant on the provision
532 of adequate heating / ventilation and maintenance levels. In this instance, we found
533 that condensation increased the risk of *Aspergillus/Penicillium, Cladosporium* and
534 *Ulocladium/Alternaria/Epicoccum*, which is supported by the findings of Sharpe et al.
535 (2014c). We also found that the presence of an odour was associated with an
536 increased risk of *Cladosporium,* which is a known allergenic fungus (Simon-Nobbe et
537 al., 2008), and may explain the associations between fungal odour, lack of ventilation
538 and asthma (Hägerhed-Engman et al., 2009; Sharpe et al., 2015b; Sharpe et al.,
539 2015c). Our findings may have been influenced by the sampling method and period
540 because we found no association with moisture readings or relative humidity~~, which~~
541 ~~could be due to the sampling within warmer months and limited sample size~~. In
542 contrast to existing knowledge, increased relative humidity reduced the risk of
543 *Cladosporium,* which is an abundant outdoor fungus (Flannigan et al., 2011). This
544 may be a result of sampling within warmer months, limited sample size, limitations of
545 taking spot measurements that do not take into account of fluctuations and residents
546 opening their windows prior to the survey, which means indoor conditions reflecting
547 outdoor humidity levels (Appendix E).

548 Alternatively, the indoor microbial profile is a function of dispersal of fungal
549 spores from outdoors into the indoor environment, which varies geographically
550 (Amend et al., 2010; Vesper et al., 2011) and seasonally (de Ana et al., 2006), and
551 explains why some studies report slightly different findings with water leaks for
552 example (Gent et al., 2002). Only a subset of these organisms infiltrating the indoor
553 environment are capable of finding suitable growth conditions, which will lead to the
554 re-suspension of spores and hyphae should surfaces become contaminated with
555 fungi (Adams et al., 2013). Fungal diversity will be regulated by a number of factors
556 including the availability of organic material (Mensah-Attipoe et al., 2014a), type of
557 material (Andersen et al., 2011) and its chemical composition, pH and physical
558 properties (Verdier et al., 2014), as well as temperature and moisture levels
559 (Johansson et al., 2013), which modifies the growth of different hydrophilic and
560 xerophilic fungi (Flannigan et al., 2011). Despite these limitations, our results further
561 support the need for future energy efficiency interventions along with behavioural
562 change to create a shift in adequate heating and ventilation patterns, which is
563 required to avoid increased risk of condensation (BSI, 2011; Hamilton et al., 2015;
564 Sharpe et al., 2015b).

565 This study highlights the necessity to effectively remediate water leaks, rising
566 damp and condensation in properties. These measures must be delivered along with
567 the provision of measures to help alleviate the impact of fuel poverty on vulnerable
568 populations, which increases the risk of visible fungal contamination and odour
569 regardless of the use of ventilation (Sharpe et al., 2015a). Failure to include fiscal
570 incentives / help and/or occupant awareness and educational initiatives may explain
571 why fungal growth sometimes returns following housing interventions (Richardson et
572 al., 2005) and why the use of mechanical ventilation failed to reduce allergen levels

573 in a previous study (Wright et al., 2009). A larger study population and the
574 development of quantitative sampling using the mAbs is required to assess how
575 different occupant behaviour's and the built environment regulate fungal diversity in
576 order to identify cost-effective measures to help prevent the reoccurrence of fungal
577 growth. Developments in diagnostic technologies to identify and quantify fungal
578 species will help improve our understanding of the potential health risks resulting
579 from variations in fungal diversity (Pringle, 2013), and help develop a cost-effective
580 approach (Méheust et al., 2013) to further our understanding into factors regulating
581 fungal diversity.

582 Strengths of our study include an interdisciplinary approach that uses asset
583 management and molecular and epidemiological techniques to investigate the
584 relationship between energy efficiency and risk of allergenic fungi. We observed high
585 correlation between the results of fungal identification using mAb-based ELISAs and
586 genus/species identification based on ITS sequencing. We also used face-to-face
587 questionnaires to obtain demographic, health and behavioural data, which may
588 reduce bias when compared to self-reported questionnaires, although both methods
589 have been found to correlate well with building inspections (Hernberg et al., 2014).
590 We also found that the study representativeness was similar to the target population
591 (i.e. all those residing in the social housing properties), but a larger sample size is
592 required to improve our confidence in these results. We conducted home inspections
593 following a standard template, which was designed using previous best practice
594 (Flannigan et al., 2011), and a moisture meter to assess risk of dampness as set out
595 by the British Standards Institution (BSI, 2011). The Index of Multiple Deprivation
596 (IMD) score was used because it has been shown to have a strong relation with

597 health in both rural and urban areas (Jordan et al., 2004), and found to be associated
598 with increased risk of fungal contamination (Sharpe et al., 2015a).

599 A number of limitations exist. Our limited sample size and cross sectional study
600 design prevented us from assessing natural fluctuations in dampness and fungal
601 contamination. We did not quantify air-borne or dust-borne concentrations in indoor
602 and outdoor environments, and were unable (with the mAbs) to distinguish between
603 similarly related fungi such as *Aspergillus* and *Penicillium* spp. which differ in
604 physiology, ecology and significance for health (Flannigan et al., 2011). The
605 prevalence of dampness problems and asthma / allergy in this study is higher than
606 the UK National average of around 10% (Court et al., 2002) and 16% (Haverinen-
607 Shaughnessy, 2012), respectively. This may be due to participants having a lower
608 SES than the UK population and/or bias due to confounding factors associated with
609 the tendency to not respond to questionnaires / surveys. Alternatively those with
610 current damp / fungal contamination and/or health problems may have been more
611 likely to participate (Sharpe, 2015). Also, the potential of selection bias may influence
612 estimates when response rates fall below 62% (Rönmark et al., 2009), which may be
613 compounded by young male participants and current smokers found to typically not
614 respond to questionnaires (Kotaniemi et al., 2001). The methodology does not
615 involve quantitative PCR to assess indoor fungi, making it impossible to compare our
616 findings with the diversity and concentrations of air or dust borne fungi. The diversity
617 of indoor fungi (i.e. the number of isolates) is relatively low as we only extracted 204
618 isolates from samples taken from 41 homes, which may be due to our sampling
619 method (only swabbing of contaminated sites).

620 In conclusion, the combined use of culture and highly specific mAbs offers an
621 alternative technique to PCR for assessing risk factors promoting the growth of

622 culturable allergenic fungi known to be associated with respiratory diseases in
623 humans. Increased energy efficiency may lower the risk of fungal contamination with
624 *Aspergillus/Penicillium* and *Cladosporium* spp (when combined with measures to
625 prevent condensation and/or water ingress). Home improvements must be delivered
626 alongside changes in occupant behaviour to address the corresponding reduction in
627 ventilation rates when homes are sealed to prevent heat loss. A larger sample and
628 continuous monitoring of the diversity and concentrations of fungi in indoor/outdoor
629 environments will improve our understanding into factors regulating their growth and
630 impact on health.
631
632 **Supporting information**
633
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640
641 **Conflict of Interest**
642 We declare that none of the authors involved in writing this paper have any conflict of
643 interests with respect to the content of this article.

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Identifying risk factors for exposure to culturable allergenic moulds in energy efficient homes by using highly specific monoclonal antibodies

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Abstract

The aim of this study was to determine the accuracy of monoclonal antibodies (mAbs) in identifying culturable allergenic fungi present in visible mould growth in energy efficient homes, and to identify risk factors for exposure to these known allergenic fungi. Swabs were taken from fungal contaminated surfaces and culturable yeasts and moulds isolated by using mycological culture. Soluble antigens from cultures were tested by ELISA using mAbs specific to the culturable allergenic fungi *Aspergillus* and *Penicillium* spp., *Ulocladium*, *Alternaria,* and *Epicoccum* spp., *Cladosporium* spp., *Fusarium* spp., and *Trichoderma* spp. Diagnostic accuracies of the ELISA tests were determined by sequencing of the internally transcribed spacer 1 (ITS1)-5.8S-ITS2-encoding regions of recovered fungi following ELISA. There was 100% concordance between the two methods, with ELISAs providing genus-level identity and ITS sequencing providing species-level identities (210 out of 210 tested). Species of *Aspergillus/Penicillium, Cladosporium, Ulocladium/Alternaria/Epicoccum, Fusarium* and *Trichoderma* were detected in 82% of the samples. The presence of condensation was associated with an increased risk of surfaces being contaminated by *Aspergillus/Penicillium* spp. and *Cladosporium* spp., whereas moisture within the building fabric (water ingress/rising damp) was only associated with increased risk of *Aspergillus/Penicillium* spp. Property type and energy efficiency levels were found to moderate the risk of indoor surfaces becoming contaminated with *Aspergillus/Penicillium* and *Cladosporium* which in turn was modified by the presence of condensation, water ingress and rising damp, consistent with previous literature.

Key words*:* Allergenic fungi, asthma, monoclonal antibody, antigen, ELISA

Ethical Approval

Ethical approval for this cross sectional study was granted by the University of Exeter Medical School, application number 13/02/013.

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Abbreviations

ELISA:	Enzyme-Linked Immunosorbent Assay
IAQ:	Indoor air quality
mAb:	Monoclonal antibody
OR:	Odds ratio
SAP:	Standard assessment procedure
VOC:	Volatile organic compound

Introduction

Increased exposure to indoor damp and associated fungal contamination is a worldwide public health concern because of its association with an increased risk of allergic diseases (Fisk et al., 2007; Mendell, 2014; Quansah et al., 2012), now present in around a third of the European population (Annesi-Maesano and Moreau, 2009). Fungal growth on surfaces in homes increases resident's exposures to elevated concentrations of spores and hyphal fragments (Sharpe et al., 2014c), which in turn is influenced by the type of material (Andersen et al., 2011), moisture (Flannigan et al., 2011), indoor air velocity, and the types of fungi present (Mensah-Attipoe et al., 2014b). There is limited research assessing how the interaction between occupant behaviours and the built environment regulates the diversity of allergenic fungi (Sharpe et al., 2014b). This is important to consider because different genera of allergenic fungi are associated with the development (Reponen et al., 2011) and exacerbation of asthma (Sharpe et al., 2014b), and a phenotype of severe asthma in sensitised individuals (Denning et al., 2014; Denning et al., 2006). Despite current knowledge of the involvement of fungal allergens in the pathophysiology of allergic diseases, fungi as a prominent source of allergens are still largely neglected(Crameri et al., 2013).

Culturability of fungal propagules has a profound effect on the production of allergens, with culturable spores having a greater potential to evoke inflammatory disease than dead ones when deposited in the respiratory tract (Lee et al., 2006; Sercombe et al., 2004). Furthermore, increased allergen production during spore germination has been demonstrated (Green et al., 2003; Lee et al., 2006; Mitakakis et al., 2001; Sercombe et al., 2004). Consequently, methods of identification are needed that extend beyond categorisation of fungal contamination by the presence of

dampness and visible fungal growth, to detection of culturable moulds known to cause allergic reactions such as *Aspergillus* (Gravesen et al., 1999; Patterson and Strek, 2010; Shen et al., 2007), *Penicillium* (Gravesen et al., 1999; Shen et al., 2007), *Ulocladium* (Gravesen et al., 1999; Kaur et al., 2010), *Alternaria* (Breitenbach and Simon-Nobbe, 2002), *Epicoccum* (Bisht et al., 2000), *Cladosporium* (Breitenbach and Simon-Nobbe, 2002; Gravesen et al., 1999), *Trichoderma* (Lübeck et al., 2000), and *Fusarium* species (Verma and Gangal, 1994). Identifying risk factors that promote the growth of these allergenic fungi can inform housing interventions aimed at ameliorating disease symptoms in susceptible populations. Tailored housing improvements offer a cost-effective approach to delivering healthcare to individuals suffering from moderate to severe asthma (Edwards et al., 2011) and improving lung function of individuals residing in, for example, mould contaminated water-damaged homes (Norbäck et al., 2011).

The Environmental Relative Moldiness Index (ERMI), which encompasses a range of fungal indicator species (Vesper et al., 2007) has been adopted, albeit principally in the US, as a method for categorising the extent of indoor fungal contamination. The index has been used to determine levels of risk to fungal exposure in the home and to predict the occurrence of illness in homes (Vesper et al., 2006). Based on mould-specific quantitative PCR (MSQPCR), it determines loads of fungal DNA in dust samples and is being increasingly used because of its low detection limit and high specificity (Méheust et al., 2013). While MSQPCR is precise, it is based on nucleic acid-based detection methods that are unable to differentiate between DNA derived from live and dead propagules. Furthermore, the US Environment Protection Agency has not validated or peer reviewed MSQPCR or

ERMI for public use, considering it to be a research tool only, despite firms offering remediation services based on results of ERMI surveys.

No studies have investigated the combined use of culture and well-characterised fungal-specific monoclonal antibodies (mAbs) as a means of detecting and identifying culturable allergenic fungi indoors, or to use this approach to determine potential risk factors that regulate their occurrence in homes. In this study, we combine asset management, epidemiology, detection using mAb-based ELISA and validation using Internal Transcribed Spacer (ITS) sequencing of fungi, to determine potential risk factors that promote the growth of culturable allergenic *Aspergillus*, *Penicillium*, *Ulocladium*, *Alternaria*, *Epicoccum*, *Cladosporium*, *Trichoderma*, and *Fusarium* spp. in energy efficient homes. This is the first time, to our knowledge, that mAbs have been used to assess how demographic and environmental factors modify the growth of these allergenic moulds.

Methodology

Study population

Ethical approval for this cross sectional study was granted by the University of Exeter Medical School, application number 13/02/013. The Cornish Health project was conducted during 2013 and 2014 in collaboration with a social housing association located in the SW of Cornwall, England which manages around 4,000 social housing properties (Sharpe et al., 2015b). We worked closely with the social housing associations customer services contact centre to recruit study participants from the target population (customers of the social housing association) (Sharpe et al., 2015b). Using a standard template (Appendix A), customers from 83 social housing properties (those who contacted customer services between April and September 2013) were randomly selected and asked whether they wished to participate in the Cornish Health project. Interested participants were subsequently sent a covering letter and information sheets, and were then contacted by telephone five days after the postage date of each letter to arrange a home visit. Written consent was obtained using a form containing a series of scripted questions concerning participant involvement in various elements of the study. We used face-to-face questionnaires to collect demographic, behavioural and health data from participating adults (Appendix B), which was followed by an environmental survey using a standardised template (Appendix C).

Property data

Property records from the social housing association were obtained from the asset management and stock condition database in February 2014 and merged using a unique household identifier. Data included residency period, property age and build type, type of heating, glazing, insulation levels, energy efficiency ratings and date of

any property upgrades. Energy efficiency ratings were calculated according to the Government's Standard Assessment Procedure (SAP). SAP 2009 was used for compliance with building regulations in England & Wales (BRE, 2013) for new builds (Part L1A) and existing buildings (Part L1B). It is the chosen methodology for delivering the EU performance of building directive (EPBD) and is used in the calculation and creation of Energy Performance Certificates (Kelly et al., 2012). SAP is calculated for both new and existing builds, and ranges from 0 to 120 with 120 representing the highest energy efficiency rating. SAP ratings were provided by the social housing provider and were auto-assessed using RDsap 9.91 (BRE, 2014) and taken from new build energy assessments (Department of Energy & Climate Change, 2014).

Socio-economic status (SES)

We obtained the IMD scores for 32,482 LSOAs (Large Super Output Areas) in England and Wales: each area contain a mean population of between 1,000 and 1,500 people (ONS, 2014). The score uses the English Indices of Deprivation 2010 to identify areas of England experiencing multiple aspects of deprivation, and were merged with our data using property full postcodes.

Questionnaire data

Questionnaires were designed to collect data on participant demographics on all occupants and environmental exposures thought to influence the risk of asthma initiation and/or exacerbation (Dales et al., 2008; Gaffin and Phipatanakul, 2009). Boxes were provided for either partner in the household to provide answers (Appendix B). Questions covered participant age, sex, height, weight; smoking status; employment; cleaning regimes; number of rooms carpeted; pets; health data on asthma, allergy and chronic bronchitis or emphysema; heating / ventilation

regimes and whether participants thought damp/mould impacted their family's health. We modified the LARES project questionnaire (Ormandy, 2009) and ISAACs definitions (Asher et al., 1995) to assess the exacerbation of wheeze, and then current asthma by asking participants if they had seen a doctor in the last 12 months and/or take medication for asthma.

Environmental data

Home surveys were conducted throughout the year with 10, 5, 3, 10, 2, 2, 2 and 7 visits being carried out during April, May, June, July, May, September, December and January 2013/14, respectively. A trained investigator (RS) carried out environmental surveys using a Protimeter MMS2 damp meter Model: BLD8800 (General Electric, MA, US), which was calibrated according to the manufacturer's settings. Visual inspections were made to identify areas of condensation, water leakages and rising damp. Indoor ambient air temperature (^{o}C), relative humidity (%), dew point temperature (^{o}C) and vapour pressure (kPa) readings were recorded from each room surveyed. We also collected the same readings from directly outside each property. High moisture generating properties were assessed by calculating excess vapour pressure (indoor minus outdoor vapour pressure), where a limit of 0.6 kPa was set in accordance to the British Standard BS 5250:2011 (BSI, 2011). The following measurements and limits were set in accordance to the protimeter manufacturer guidelines. Risk of condensation was measured by the difference (T Diff ^{o}C), which is the difference between ambient dew point temperature (±0.3^{o}C) and the external wall surface temperature (^{o}C) of each room surveyed. In accordance to the manufacturer's meter settings and guidelines, we categorised T Diff as ≤0°C =condensation, >0 to ≤3°C = risk of condensation and >3°C no risk of condensation. Wall dampness was assessed using a non-invasive probe measuring relative

moisture at 15 mm (two readings taken at 1m intervals from the top of the skirting boards), which ranged from 60 (dry) to high moisture content (999) relative scale. Wall dampness was categorised as <170 = dry wall, ≥170 but <200 = risk of damp and ≥ 200 = dampness. Relative humidity (≥65%), vapour pressure (>1 kPa), wall surface temperature difference of <3^{0}C (TDiff) and visual signs of dampness were used as dichotomous exposure variables.

Fungal sample plan

In parallel to the collection of environmental data, contaminated surfaces with visible fungal growth were identified and selected for sampling via a home walk through with each participant and from the environmental survey. We planned to take a single sample from each surface with visible fungal growth in a home (i.e. all individual contaminated surfaces were sampled). When there was more than one surface with visible fungal growth in a room or hallway, we extracted a single sample from each contaminated site. These locations were along the window recess, along the ceiling / wall junction and floor / wall junctions and in isolated locations with water damage (leaks or rising damp). We obtained a single sample from each surface (i.e. a wall, ceiling or floor area) in severe cases where whole surfaces had signs of condensation and visible fungal growth. Samples were collected from contaminated surfaces located in the bathroom, main bedroom, child's bedroom, the hall way, kitchen, landing, living room and utility, making up 21.9% (n=46), 30.5% (n=64), 12.9% (n=27), 6.2% (n=13), 5.7% (n=12), 1% (n=2), 19.5% (n=41) and 2.4% (n=5) of the total samples, respectively. Details of the number of samples taken from each property surveyed, and within each room are provided in Appendix E. The location, clustering and number of samples taken from each room and home are accounted for in our statistical analysis plan described below.

237 **Fungal sampling within the home**

238 Surface swabs were taken using sterile cotton buds wetted with sterilised water. For
239 each of the rooms surveyed, lawns of fungal debris were prepared on-site by gently
240 stroking the swabs across the surface of malt extract agar (MEA) culture plates
241 containing the broad-spectrum antibiotic rifampicin (MP Biomedicals). After
242 transportation to the laboratory (within 48 hours), the plates were incubated under a
243 16 h fluorescent light regime at 26°C. Fungi were separated on the basis of gross
244 morphological characteristics and sub-cultured on MEA as axenic cultures.

245 **Growth of fungi and preparation of antigens**

246 For antibody specificity tests, fungi were grown as two replicate MEA slope cultures
247 and surface washings containing soluble antigens were prepared using phosphate
248 buffered saline (PBS: 0.8% NaCl; 0.02% KCl; 0.115% Na_2HPO_4; 0.02% KH_2PO_4;
249 pH7.2) as described in Thornton (2001). Protein concentrations, determined
250 spectrophotometrically at 280 nm (Nanodrop, Agilent Technologies Limited,
251 Berkshire, UK), were adjusted to 60 µg ml^{-1} buffer. Fifty-µl volumes were then used to
252 coat the wells of Maxisorp microtitre plates (Nunc DIS-971; Thermo Fisher Scientific,
253 Leicestershire). After incubating overnight at 4°C, wells were washed four times with
254 PBST (PBS containing Tween-20, 0.05% (v/v)) and once each with PBS and dH_2O
255 and air-dried at 23°C in a laminar flow hood. The plates were stored in sealed plastic
256 bags at 4°C prior to ELISA tests.

257 **Enzyme-Linked Immunosorbent Assay**

258 Wells containing immobilised antigens were blocked for 10 min with PBS containing
259 1% (w/v) bovine serum albumin (A-2153; Sigma Chemical Company, Poole, United
260 Kingdom) and then incubated for 1 h with hybridoma tissue culture supernatants
261 containing fungus-specific monoclonal antibodies (mAbs). The mAbs used were mAb

262 JF5 (mouse IgG3 specific to *Aspergillus* and *Penicillium* spp. (Thornton, 2008)), mAb
263 ED7 (mouse IgM specific to *Fusarium* spp. Thornton and Wills, 2015), mAb MF2
264 (mouse IgM specific to *Trichoderma* spp. (Thornton et al., 2002)), mAb OX-CH1
265 (mouse IgM specific to *Cladosporium* spp. (Karpovich-Tate et al., 1998)), and mAb
266 PC3 (rat IgG2a specific to *Ulocladium*, *Alternaria*, and *Epicoccum* spp. (Karpovich-
267 Tate and Dewey, 2001)). After washing four times (5 min each time) with PBST, wells
268 were incubated with goat anti-mouse polyvalent (immunoglobulin classes IgG, IgA,
269 and IgM) peroxidase conjugate (A-0412; Sigma)(mAbs JF5, ED7, MF2, OX-CH1) or
270 goat anti-rat peroxidase conjugate (G-8154; Sigma)(mAb PC3), both diluted 1 in
271 1000 in PBST containing 0.5% (w/v) BSA, for a further hour. Wells were rinsed four
272 times with PBST, once with PBS and bound antibody was visualised by incubating
273 wells with tetramethyl benzidine (T-2885; Sigma) substrate solution (Thornton, 2001)
274 for 30 min. The reactions were stopped by the addition of 3M H_2SO_4. Absorbance
275 values were determined at 450 nm with an automated microplate reader (Dynex
276 Technologies, Billingshurst, UK). Working volumes were 50 µl per well, control wells
277 were incubated with tissue culture medium (TCM) containing 10% (v/v) foetal bovine
278 serum, and incubation steps were performed at 23°C in sealed plastic bags.

Identification of fungi by analysis of the ITS regions of the rRNA-encoding gene unit

Representative isolates of antibody-reactive and antibody non-reactive fungi recovered from the 32 households were used to determine the accuracy of the mAb-based ELISA tests. Species were identified by sequencing of the ITS1-5.8S-ITS2 region of the rRNA-encoding gene unit (White et al., 1990) according to procedures described elsewhere (Thornton et al., 2002) using the primers ITS1ext (5'-GTAACAAGGTTTCCGTAGGTG-3') and ITS4ext (5'TTCTTTTCCTCCGCTTATTGATATGC-3'). Newly determined sequences were submitted to GenBank, and accession numbers KP794062 to KP794197 were obtained. Species designations of recovered fungi are shown in Appendix D.

Statistical analysis

Aspergillus/*Penicillium*, *Ulocladium*/*Alternaria*/*Epicoccum*, and *Cladosporium* groupings identified using the mAbs were used as our dichotomous outcome variables for statistical analysis because we were interested in factors regulating their presence or absence on contaminated surfaces. Due to a small sample size, *Fusarium* and *Trichoderma* were omitted from these analyses. Our small sample size also prevented us from developing multiple environmental categories or continuous variables such as variations in temperature, humidity and vapour pressure. Behavioural, built environment and survey data previously described as dichotomous exposure variables were also used in our analyses. Descriptive statistics were used to depict the demographic and housing characteristics of participating homes. In order to help reduce the impact of multiple testing, we developed a detailed analysis plan to investigate the demographic and built environment risk factors. In our accordance to our analysis plan Pearson's chi-squared tests were used to assess

differences between our exposure and outcome variables of interest. We adopted a survey methodology that resulted in multiple samples being taken from each house with visible fungal growth. For this reason we used multilevel mixed-effects logistic regression (fixing each household surveyed and the location of each sample) to account for residual variance that may occur between groupings of fungal samples, which was carried out using the meqrlogit command in Stata version 13.0 (Stata Corp., College Station, US). We used an *a priori* in our adjusted models, which included occupancy rates, outdoor ambient air temperature and whether households said they ventilated to minimise dampness and fungal growth because these have been found to modify the risk of fungal growth. Both chi-squared tests and logistic regression was used to assess whether 1) signs of dampness, condensation and fungal odour, 2) increased household energy efficiency and 3) behavioural/housing characteristics, increased the risk of indoor surfaces being contaminated with *Aspergillus*/*Penicillium*, *Ulocladium*/*Alternaria*/*Epicoccum*, or *Cladosporium* spp.

Results

Study characteristics

The study achieved a response rate of 49% and the results are based on 41 participating households and 93 inhabitants. Children had a slightly higher prevalence of doctor diagnosed asthma (30%) and allergy (18%) when compared to adults (24% and 16%, respectively). A high proportion of homes had visible fungal growth (78%) and the presence of a fungal odour (37%). Participant behaviours and building characteristics varied (Table 1). Mean air temperature, relative humidity, dew point temperature and vapour pressure readings taken from living rooms and main bedrooms were slightly greater than ambient levels taken from outside each property. Sixty per cent of rooms had windows open, and mean external wall surface temperatures were greater than dew point temperatures, although homes had varying levels of dampness related problems and the majority of homes had their chimneys sealed with air vents (Table 2). The demographics of our target population (those living in homes owned and managed by the social housing provider) differed slightly from our study participants (Table 1) in terms of a mean age (36 versus 59 years), proportion of male residents (44 versus 41%) and occupancy rates (2.3 versus 1.7 persons per house). Household demographics of target homes were similar to participating homes in terms of their IMD score (mean 28.4 versus 34.1), build age (1967 versus 1968), energy efficiency (SAP 66.0 versus 65.7) and use of gas heating (53 versus 55%) (Sharpe et al., 2015b).

Use of monoclonal antibodies to identify fungi and confirmation of antibody specificities by using ITS sequencing

Five specific mAbs were used to identify fungal genera in ELISA tests of crude antigen extracts from axenic fungal cultures. All five of the mAbs were shown to be specific for their target species (Appendix D). Of the 204 fungal isolates tested by ELISA (i.e. all fungal isolates taken from the original surface swabs of visible fungal growth in participating homes), 40% reacted with mAb JF5 (specific for *Aspergillus* and *Penicillium* spp.), 27% reacted with mAb CF1 (specific for *Cladosporium* spp.), 9% reacted with mAb PC3 (specific for *Ulocladium*, *Alternaria*, *and Epicoccum* spp.), 3% reacted with mAb ED7 (specific for *Fusarium* spp.), and 3% reacted with mAb MF2 (specific for *Hypocrea* (*Trichoderma*) spp.). The remaining fungi were unrelated species non-reactive with the five mAbs tested. ITS sequencing of 136 of the fungal strains confirmed antibody specificities, with mAb JF5 reacting specifically with strains of *Aspergillus candidus*, *A. flavus*, *A. oryzae*, *A. tennesseensis*, *A. tubigensis*, *A. versicolor*, and strains of *Penicillium brevicompactum*, *P. chrysogenum*, *P. concentricum*, *P. commune*, *P. copticola*, *P. corylophilum*, *P. crustosum*, *P. expansum*, *P. glabrum*, *P. polonicum*, *P. toxicarium*, and *P. tricola*. Fungi reactive with mAb CF1 were identified as *Cladosporium cladosporioides*, *C. lignicola, C. ossifragi*, and *C. sphaerospermum*. Fungi reactive with mAb ED7 were identified as *Fusarium oxysporum* and *F. solani*, while fungi reactive with mAb MF2 were identified as teleomorphic or anamorphic *Trichoderma* spp. including *Hypocrea atroviridis* (anamorph *T. atroviride*), *H. lixii* (anamorph *T. harzianum*), *H. viridescens* (anamorph *T. viridescens*) and *T. viride*. Fungi reactive with mAb PC3 included the target species *Alternaria alternata*, *Epicoccum nigrum*, and *Ulocladium obovoideum*, and *Pyrenochaeta unguis-hominis*, a dematiaceous fungus belonging to the

376 mitosporic Pleosporaceae group that includes the genera *Alternaria* and *Ulocladium*
377 (Liu, 2011). The antibody also cross-reacted with a single isolate of the unrelated
378 allergenic fungus *Phoma herbarum*. None of the mAbs cross-reacted with the
379 unrelated fungi identified using ITS sequencing including the moulds *Acremonium*
380 *sclerotigenum*, *Bjerkandera adusta*, *Eutypa lata*, *Nectria maurtitiicola*, *Peniophora*
381 *lycii*, *Periconia byssoides*, *Pseudeurotium bakeri*, the yeasts *Candida famata*
382 (*Debaryomyces hansenii*), *Candida intermedia*, *Candida parapsilosis*, *Candida*
383 *spencermartinsiae*, *Cryptococcus diffluens*, *Meyerozyma guilliermondii*, *Rhodotorula*
384 *mucilaginosa*, or the yeast-like fungus *Aureobasidium pullulans*.

385 **Indoor condensation and presence of an odour**

386 We assessed whether the presence of condensation and odour were risk factors for
387 the target genera of interest. Signs of condensation increased the risk of surfaces
388 being contaminated with *Aspergillus*/*Penicillium* (OR 2.37; 95% CI 1.05-5.36) and
389 *Cladosporium* (OR 4.32; 95% CI 1.23-15.20) in the adjusted models, but not
390 *Ulocladium*/*Alternaria*/*Epicoccum* (Table 3). On further investigation the presence of
391 condensation increased the risk of all three groups of fungus when we combined
392 them into unadjusted models (OR 2.85; 95% CI 1.15-7.10) and adjusted models (OR
393 2.52 95% CI 1.06-5.99). Only *Aspergillus*/*Penicillium* was associated with signs of
394 water damage (plumbing leakages) and rising damp in properties surveyed (OR 2.08;
395 95% CI 1.02-4.23). The presence of a fungal odour was associated with increased
396 risk of *Cladosporium* in the unadjusted model (OR 2.96; 95% CI 1.19-7.32), but not in
397 the adjusted model. No association was observed with odour and
398 *Aspergillus*/*Penicillium* and *Ulocladium*/*Alternaria*/*Epicoccum.* We observed no
399 association between mean wall moisture readings (two taken at 1 m intervals to

assess water ingress in the building fabric) or other dampness measures (wall surface temperature and vapour pressure) and risk of either fungus.

Aspergillus/*Penicillium* and *Ulocladium*/*Alternaria*/*Epicoccum* were not associated with elevated relative humidity, but high relative humidity was inversely associated with risk of *Cladosporium* (OR 0.40; 95% CI 0.17-0.91). On further investigation (Appendix E) we found that mean relative humidity was similar in ventilated and unventilated homes, we observed higher vapour pressure readings (indoor minus outdoor readings) in unventilated homes. However, mean values did not exceed those typically found in a UK property (0.5 to 0.6 kPa) during the winter months (BSI, 2011).

Energy efficiency and risk of allergenic fungi

Increased household energy efficiency was inversely associated with *Aspergillus*/*Penicillium* contamination in unadjusted models when SAP was between ≥61-69 (OR 0.43; 95% CI 0.19-0.96), but not when SAP ≥69-81 (OR 0.64; 95% CI 0.31-1.30). Increased energy efficiency was inversely associated with risk of *Cladosporium* contamination when SAP ≥66-69 (OR 0.39; 95% CI 0.20-0.76) and ≥69-81 (OR 0.38 95% CI 0.16-0.87) in adjusted models. There was suggestive evidence that high SAP was associated with increased risk of *Ulocladium*/*Alternaria*/*Epicoccum* contamination (P=0.04) (Table 4). In additional analyses, signs of condensation was associated with an increased risk of surfaces being contaminated by *Cladosporium* contamination in homes with a SAP ≥66 (P<0.05), but not in low energy efficient homes. We also observed that properties with a water leak or rising damp problems were associated with increased risk of *Aspergillus/Penicillium* and *Cladosporium* in homes with a SAP ≥66 (P<0.05), but not in low energy efficient homes. We observed no association with either model

between water leaks or rising damp and *Ulocladium*/*Alternaria*/*Epicoccum* contamination.

Demographic and housing characteristics and risk of fungus

We assessed behavioural and built environment risk factors and the presence of *Aspergillus*/*Penicillium* and *Cladosporium,* but excluded *Ulocladium*/*Alternaria*/*Epicoccum* due to the lack of power in the analyses. Participant homes that had been vacuumed prior to the home survey was inversely associated with *Aspergillus*/*Penicillium* (OR 0.46; 95% CI 0.21-0.98). Only building architecture appeared to modify the risk of fungus contaminating indoor surfaces, with a reduced (OR 0.42; 95% CI0.22-0.81) and increased (OR 3.56; 95% CI 1.41-9.05) risk of surfaces being contaminated by *Aspergillus*/*Penicillium* and *Cladosporium* spp., respectively (Tables 5 & 6).

Discussion

This is the first time to our knowledge that highly specific monoclonal antibodies (mAbs) have been used to track culturable allergenic fungi contaminating the indoor surfaces of social housing in the UK. Because of the intermittent nature of airborne spore production (Bush and Portnoy, 2001), we chose to swab contaminated surfaces directly and to use mycological culture for fungal isolation. By combining mycological culture with highly specific mAbs we were able to detect specific groups of fungi known to be involved in human allergic diseases (Sharpe et al., 2014a). While the mAb-based ELISA method is qualitative, detecting fungal presence rather than amount, its specificity (confirmed by ITS sequencing of recovered fungi) and combination with culture allows identification of culturable allergenic fungi in contrast to current MSQPCR DNA detection methods that are unable to differentiate between live and dead fungal biomass. The use of mAbs that detect signature antigens unique to these fungi removes the need for skilled identification based on morphological characteristics (Méheust et al., 2013). While the mAbs used in this study were unable to identify the fungi to species level, their accuracy in detecting individual genera (*Cladosporium*, *Fusarium* and *Trichoderma*) or genus-level groupings (*Aspergillus*/*Penicillium* and *Ulocladium*/*Alternaria*/*Epicoccum*), meant that culturable allergenic fungi known to be involved in human respiratory diseases (Denning et al., 2014; Simon-Nobbe et al., 2008; Thornton C.R and Wills O.E, 2015) could be detected with a high degree of accuracy by using crude antigen extracts. Indeed, four of the mAbs displayed 100% specificity for their target species, while mAb PC3 targeting *Alternaria*, *Epicoccum*, and *Ulocladium* cross-reacted with only a single strain of *Phoma*, a genus not previously tested with this mAb [43]. A major advantage of mAbs is their adaptability to field-based diagnostics such as lateral-flow assays

(LFA). To this end, mAb JF5 has been used to develop a commercially available LFA for point-of-care diagnosis of invasive pulmonary aspergillosis in immunocompromised patients (Thornton, 2008; Prattes et al., 2014). Similar LFAs incorporating the other mAbs described here could be used as monitoring tools to track contamination by known allergenic fungi. The speed, low cost and simplicity of such assays compared to relatively expensive and sophisticated laboratory-based DNA identification methods could simplify the risk assessment process.

The ELISAs represent a significant improvement on diagnostic procedures based on pan-fungal cell wall constituents such as ergosterol and β(1✶3)-glucan that are unable to discriminate between fungi and which can be affected by non-fungal sources (Bush and Portnoy, 2001). Other immunoassays that have been used to monitor fungal exposure are based on allergen detection in airborne or settled dust samples. While these are able to detect single allergenic proteins they can, in some instances, be too specific, being unable to detect the presence of fungi that do not produce the precise allergens that the assays are designed to detect (Bush and Portnoy, 2001). Our method is not allergen-specific (i.e. not all of the species detected using the mAbs are allergenic). Consequently, immunoassays such as those described here that identify fungi directly using signature antigens, and those that detect the fungi indirectly using allergenic proteins, could be combined to provide a more accurate measure of exposure to allergenic species in the indoor environment.

Using the mAb-based ELISAs, *Aspergillus*/*Penicillium*, *Cladosporium*, *Ulocladium*/*Alternaria*/*Epicoccum*, *Fusarium*, and *Trichoderma* spp. were shown to constitute 82% of the fungal species recovered from contaminated surfaces with areas of visible fungal growth. While the presence of these fungi in the home may

represent a respiratory health risk in susceptible individuals (Sharpe et al., 2014b), further research is required to assess and compare exposures to elevated concentration of these fungi, as well as the impact of other fungi not assessed in this study. Nearly half of the fungal isolates were collected from bedrooms within the properties surveyed, where occupants spend the majority (~8 hours) of their time. *Aspergillus*/*Penicillium* and *Cladosporium* were the most frequent fungi identified, which corresponds to other studies (Flannigan et al., 2011). Flannigan et al. (2011) reported that spores of *Aspergillus*/*Penicillium* and *Cladosporium* were the most abundant types in indoor air in US homes, making up 19.8% and 38.8% of the total aerospora respectively, with *Aspergillus*/*Penicillium* exceeding outdoor concentrations. These fungi can dominate indoor environments, where they have been cultured from sputum of asthmatic and non-asthmatic individuals, and implicated in the initiation or exacerbation of asthma (Agbetile et al., 2012). The identification and control of these high risk fungi may help to improve health and benefit asthmatic individuals (Meng et al., 2012), although the complex interaction between occupant behaviours and the built environment must also be considered (Figure 1).

In keeping with our findings, other studies have found that fungal diversity (number and type of fungal genera) is modified by factors such as occupancy patterns (Howden-Chapman et al., 2005), opening windows or use of extractor fans (Dharmage et al., 1999; Zock et al., 2002), property type, age, architecture and, presence of carpeting / vacuuming, build age and type (Chew et al., 2003; Dharmage et al., 1999; Fairs et al., 2010; Reponen et al., 2013; Zock et al., 2002), type of dampness problem (Gent et al., 2002; Zock et al., 2002), sun exposure (Howden-Chapman et al., 2005), type of heating / ventilation (Dharmage et al., 1999; Fairs et

al., 2010) and the extent of household insulation (Semple et al., 2012). Reduced risk of cold bridging (BSI, 2011) explains why increased household energy efficiency reduced the risk of surfaces being contaminated by *Aspergillus*/*Penicillium* and *Cladosporium* spp. Energy efficiency interventions have been found to lower the risk of visible fungal growth (Sharpe et al., 2015b), although this is reliant on the provision of adequate heating / ventilation and maintenance levels. In this instance, we found that condensation increased the risk of *Aspergillus/Penicillium, Cladosporium* and *Ulocladium/Alternaria/Epicoccum*, which is supported by the findings of Sharpe et al. (2014c). We also found that the presence of an odour was associated with an increased risk of *Cladosporium,* which is a known allergenic fungus (Simon-Nobbe et al., 2008), and may explain the associations between fungal odour, lack of ventilation and asthma (Hägerhed-Engman et al., 2009; Sharpe et al., 2015b; Sharpe et al., 2015c). Our findings may have been influenced by the sampling method and period because we found no association with moisture readings or relative humidity. In contrast to existing knowledge, increased relative humidity reduced the risk of *Cladosporium,* which is an abundant outdoor fungus (Flannigan et al., 2011). This may be a result of sampling within warmer months, limited sample size, limitations of taking spot measurements that do not take into account of fluctuations and residents opening their windows prior to the survey, which means indoor conditions reflecting outdoor humidity levels (Appendix E).Alternatively, the indoor microbial profile is a function of dispersal of fungal spores from outdoors into the indoor environment, which varies geographically (Amend et al., 2010; Vesper et al., 2011) and seasonally (de Ana et al., 2006), and explains why some studies report slightly different findings with water leaks for example (Gent et al., 2002). Only a subset of these organisms infiltrating the indoor environment are capable of finding suitable growth conditions,

which will lead to the re-suspension of spores and hyphae should surfaces become contaminated with fungi (Adams et al., 2013). Fungal diversity will be regulated by a number of factors including the availability of organic material (Mensah-Attipoe et al., 2014a), type of material (Andersen et al., 2011) and its chemical composition, pH and physical properties (Verdier et al., 2014), as well as temperature and moisture levels (Johansson et al., 2013), which modifies the growth of different hydrophilic and xerophilic fungi (Flannigan et al., 2011). Despite these limitations, our results further support the need for future energy efficiency interventions along with behavioural change to create a shift in adequate heating and ventilation patterns, which is required to avoid increased risk of condensation (BSI, 2011; Hamilton et al., 2015; Sharpe et al., 2015b).

This study highlights the necessity to effectively remediate water leaks, rising damp and condensation in properties. These measures must be delivered along with the provision of measures to help alleviate the impact of fuel poverty on vulnerable populations, which increases the risk of visible fungal contamination and odour regardless of the use of ventilation (Sharpe et al., 2015a). Failure to include fiscal incentives / help and/or occupant awareness and educational initiatives may explain why fungal growth sometimes returns following housing interventions (Richardson et al., 2005) and why the use of mechanical ventilation failed to reduce allergen levels in a previous study (Wright et al., 2009). A larger study population and the development of quantitative sampling using the mAbs is required to assess how different occupant behaviour's and the built environment regulate fungal diversity in order to identify cost-effective measures to help prevent the reoccurrence of fungal growth. Developments in diagnostic technologies to identify and quantify fungal species will help improve our understanding of the potential health risks resulting

569 from variations in fungal diversity (Pringle, 2013), and help develop a cost-effective
570 approach (Méheust et al., 2013) to further our understanding into factors regulating
571 fungal diversity.

572 Strengths of our study include an interdisciplinary approach that uses asset
573 management and molecular and epidemiological techniques to investigate the
574 relationship between energy efficiency and risk of allergenic fungi. We observed high
575 correlation between the results of fungal identification using mAb-based ELISAs and
576 genus/species identification based on ITS sequencing. We also used face-to-face
577 questionnaires to obtain demographic, health and behavioural data, which may
578 reduce bias when compared to self-reported questionnaires, although both methods
579 have been found to correlate well with building inspections (Hernberg et al., 2014).
580 We also found that the study representativeness was similar to the target population
581 (i.e. all those residing in the social housing properties), but a larger sample size is
582 required to improve our confidence in these results. We conducted home inspections
583 following a standard template, which was designed using previous best practice
584 (Flannigan et al., 2011), and a moisture meter to assess risk of dampness as set out
585 by the British Standards Institution (BSI, 2011). The Index of Multiple Deprivation
586 (IMD) score was used because it has been shown to have a strong relation with
587 health in both rural and urban areas (Jordan et al., 2004), and found to be associated
588 with increased risk of fungal contamination (Sharpe et al., 2015a).

589 A number of limitations exist. Our limited sample size and cross sectional study
590 design prevented us from assessing natural fluctuations in dampness and fungal
591 contamination. We did not quantify air-borne or dust-borne concentrations in indoor
592 and outdoor environments, and were unable (with the mAbs) to distinguish between
593 similarly related fungi such as *Aspergillus* and *Penicillium* spp. which differ in

594 physiology, ecology and significance for health (Flannigan et al., 2011). The
595 prevalence of dampness problems and asthma / allergy in this study is higher than
596 the UK National average of around 10% (Court et al., 2002) and 16% (Haverinen-
597 Shaughnessy, 2012), respectively. This may be due to participants having a lower
598 SES than the UK population and/or bias due to confounding factors associated with
599 the tendency to not respond to questionnaires / surveys. Alternatively those with
600 current damp / fungal contamination and/or health problems may have been more
601 likely to participate (Sharpe, 2015). Also, the potential of selection bias may influence
602 estimates when response rates fall below 62% (Rönmark et al., 2009), which may be
603 compounded by young male participants and current smokers found to typically not
604 respond to questionnaires (Kotaniemi et al., 2001). The methodology does not
605 involve quantitative PCR to assess indoor fungi, making it impossible to compare our
606 findings with the diversity and concentrations of air or dust borne fungi. The diversity
607 of indoor fungi (i.e. the number of isolates) is relatively low as we only extracted 204
608 isolates from samples taken from 41 homes, which may be due to our sampling
609 method (only swabbing of contaminated sites).

610 In conclusion, the combined use of culture and highly specific mAbs offers an
611 alternative technique to PCR for assessing risk factors promoting the growth of
612 culturable allergenic fungi known to be associated with respiratory diseases in
613 humans. Increased energy efficiency may lower the risk of fungal contamination with
614 *Aspergillus/Penicillium* and *Cladosporium* spp (when combined with measures to
615 prevent condensation and/or water ingress). Home improvements must be delivered
616 alongside changes in occupant behaviour to address the corresponding reduction in
617 ventilation rates when homes are sealed to prevent heat loss. A larger sample and
618 continuous monitoring of the diversity and concentrations of fungi in indoor/outdoor

environments will improve our understanding into factors regulating their growth and impact on health.

Supporting information

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Conflict of Interest

We declare that none of the authors involved in writing this paper have any conflict of interests with respect to the content of this article.

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Table

Table 1 Demographic & housing characteristics

Variable	Study participants				
	n	(%)	Mean	sd	range
Summary of participant characteristics (N=93)					
Mean household occupancy	41		2.3	1.4	1-7
Mean age of household occupants	91		36	23.9	0-89
Proportion of male occupants	40/91	44			
Households with children	18/41	44			
Adults in employment or self-employed	22/61	36			
Adults smoke Adults ex-smoker	12/61 29/61	20 48			
Summary of housing characteristics (N=41)					
Mean energy efficiency, SAP rating	38		66	7.9	50-81
Presence of any pet	27/41	66			
Participants dries washing indoors	28/40	70			
Ventilate to minimise damp and fungal growth	34/41	83			
Don’t heat the home due to cost of fuel	11/40	28			
Indices of Multiple Deprivation 2010	41		28.4	12.6	9.4-58.8
Mean build age of property	40		1967	14.2	1929-2007
Property type; End terraced house Flat Mid terraced house Semi-detached house	 5/41 8/41 21/41 7/41	 12 20 51 17			
Type of heating system; Air Source Heat Pump Electric Heating Full Electric Heating Part Gas Heating Full Oil Heating Full Solid Fuel Heating Part	 2/40 3/40 1/40 21/40 10/40 3/10	 5 7 3 53 25 7			
Homes with visible mould contamination Presence of a mouldy/musty odour in last 12 months	32/41 15/41	78 37			

Table 2 Home visit and summary of environmental data collected

Variable	Study participants				
	n	(%)	Mean	sd	range
Mean outdoor readings;	41				
Ambient temperature (^{0}C)			18.5	5.4	9.5-27
Relative humidity (%)			61.1	12.1	40.8-80.7
Due point (^{0}C)			11.1	3.5	5.2-17.3
Vapour pressure (kPa)			1.3	0.3	0.9-2.04
Mean indoor readings taken from the living room;	39				
Ambient temperature (^{0}C)			19.5	3.9	11.7-27.9
Relative humidity (%)			65.2	10.8	43.9-86.6
			12.7	2.9	7.3-18.7
Dew point (^{0}C)			1.5	0.3	0.9-2.1
Vapour pressure (kPa)			18.3	2.7	12.4-25
External wall surface temperature (kPa)			5.9	2.7	0.7-10.6
Risk of condensation (T Diff)			220.0	186.3	8.2-999
Wall dampness (relative scale)	12/39	31			
Visible signs of condensation	4/39	10			
Signs of rising damp	28/39	62			
Ventilation / Windows open during survey	24/30	80			
Chimney sealed with/without air vent	8/28	29			
Decorated in last 12 months	5/39	13			
Fungal growth cleaned in last 12 months	8/39	21			
Room cluttered with furniture	9/37	24			
Visible fungal growth					
Mean indoor readings taken from the main bedroom;	37				
Ambient temperature (^{0}C)			19.9	3.3	12.5-28.2
Relative humidity (%)			63.6	7.0	46.3-76.2
Dew point (^{0}C)			12.7	2.9	6.8-19.3
Vapour pressure (kPa)			1.5	0.3	0.96-2.12
External wall surface temperature (kPa)			17.24	3.8	5.9-26.5
Risk of condensation (T Diff)			4.9	2.2	1.7-9.6
Wall dampness (relative scale)			182	45.9	7.9-3.54
Visible signs of condensation	20/37	54			
Ventilation / Windows open during survey	25/37	68			
Decorated in last 12 months	7/37	19			
Fungal growth cleaned in last 12 months	11/37	30			
Room cluttered with furniture	13/17	35			
Visible fungal growth	21/35	60			

- Some environmental data is missing from a small number of houses visited, which was due to limited access to those rooms (room containing dogs for example) and participants not willing access to their bedrooms.

Table 3 Indoor condensation, damp and the presence of self-reported fungal odour

Dampness variables	Percent (n/d)	P Value	Risk of fungus			
			Unadjusted		Adjusted∞	
			OR	95% (CI)	OR	95% (CI)
Aspergillus, Penicillium						
Visible signs of condensation; no Yes	 26 (11/43) 40 (74/183)	 0.07	 Ref 2.16	 0.92-2.08	 Ref 2.37	 1.05-5.36*
Presence of a fungal odour; no Yes	37 (37/99) 38 (49/129)	0.93	Ref 1.10	 0.56-2.15	Ref 1.66	 0.80-3.45
Signs of water leakage or rising damp; no Yes	 33 (58/174) 52 (27/52)	 0.02	 Ref 2.28	 1.10-4.70*	 Ref 2.08	 1.02-4.23*
Mean wall dampness reading; 97-170 ≥170-184 ≥184	 11 (9/83) 6 (4/70) 9 (6/71)	 0.53	 Ref 0.97 1.06	 0.44-2.13 0.48-2.31	 Ref 0.91 1.10	 0.42-1.97 0.49-2.49
Elevated relative humidity; <65% ≥65%	 34 (51/151) 46 (35/77)	 0.09	 Ref 1.65	 0.87-3.12	 Ref 1.57	 0.84-2.93
Cladosporium						
Visible signs of condensation; no Yes	 9 (4/43) 28 (52/183)	 0.00	 Ref 4.80	 1.31-17.64*	 Ref 4.32	 1.23-15.20*
Presence of a fungal odour; no Yes	15 (15/99) 33 (42/129)	0.00	Ref 2.96	 1.19-7.32*	Ref 2.33	 0.89-6.12
Signs of water leakage or rising damp; no Yes	 26 (45/174) 21 (11/52)	 0.49	 Ref 0.99	 0.36-2.74	 Ref 1.20	 0.46-3.13
Mean wall dampness reading; 97-170 ≥170-184 ≥184	 27 (22/83) 31 (22/70) 16 (11/71)	 0.08	 Ref 1.89 0.64	 0.69-5.00 0.21-1.93	 Ref 2.01 0.64	 0.76-5.32 0.21-1.95
Elevated relative humidity; <65% ≥65%	 31 (46/151) 14 (11/77)	 0.00	 Ref 0.44	 0.18-1.08	 Ref 0.40	 0.17-0.91*
Ulocladium, Alternaria, Epicoccum						
Visible signs of condensation; no Yes	 9 (4/43) 9 (16/183)	 0.94	 Ref 1.31	 0.21-7.98	 Ref 1.06	 0.17-6.63
Presence of a fungal odour; no Yes	12 (12/99) 6 (8/129)	0.12	Ref 0.52	 0.15-11.85	Ref 0.31	 0.08-1.17
Signs of water leakage or rising damp; no Yes	 10 (18/174) 4 (2/52)	 0.148	 Ref 0.31	 0.05-2.09	 Ref 0.27	 0.04-1.98
Mean wall dampness reading; 97-170 ≥170-184 ≥184	 11 (9/83) 6 (4/70) 9 (6/71)	 0.53	 Ref 0.43 0.78	 0.08-2.27 0.16-3.85	 Ref 0.47 0.89	 0.08-2.70 0.14-5.47
Elevated relative humidity; <65% ≥65%	 9 (13/151) 9 (7/77)	 0.90	 Ref 1.13	 0.28-4.53	 Ref 1.37	 0.32-5.92

∞ Adjusted model for number of occupants, outdoor temperature and whether participants stated they ventilate to minimise damp / fungal growth
* 0.01≤p<0.05, ** 0.001≤p<0.01 & *** p<0.001

Table 4 Energy efficiency measures & risk fungus

Dampness variables	Percent (n/d)	P value	Risk of fungus			
			Unadjusted		Adjusted∞	
			OR	95% (CI)	OR	95% (CI)
Aspergillus, Penicillium						
Mean energy efficiency ;						
Low SAP (<66)	41 (44/108)	0.37	Ref		Ref	
High SAP (≥66)	35 (42/120)		0.75	0.40-1.42	0.82	0.44-1.53
Energy efficiency, SAP tertiles;						
50-61	44 (38/86)	0.08	Ref		Ref	
≥61-69	26 (15/57)		0.43	0.19-0.96*	0.48	0.18-1.25
≥69-81	34 (24/71)		0.64	0.31-1.30	0.66	0.32-1.35
Cladosporium						
Mean energy efficiency ;						
Low SAP (<66)	32 (35/108)	0.01	Ref		Ref	
High SAP (≥66)	18 (22/120)		0.47	0.19-1.14	0.39	0.20-0.76*
Energy efficiency, SAP tertiles;						
50-61	30 (26/86)	0.02	Ref		Ref	
≥61-69	35 (20/57)		1.25	0.51-3.08	0.53	0.21-1.36
≥69-81	15 (11/71)		0.46	0.18-1.19	0.38	0.16-0.87*
Ulocladium, Alternaria, Epicoccum						
Mean energy efficiency ;						
Low SAP (<66)	5 (5/108)	0.04	Ref		Ref	
High SAP (≥66)	13 (15/120)		3.56	0.86-14.81	3.47	0.82-14.61
Energy efficiency, SAP tertiles;						
50-61	5 (4/86)	0.12	Ref		Ref	
≥61-69	9 (5/57)		2.30	0.35-15.14	1.87	0.22-16.09
≥69-81	14 (10/71)		4.34	0.72-25.95	4.21	0.69-25.75

∞ Adjusted model for number of occupants, outdoor temperature and whether participants stated they ventilate to minimise damp / fungal growth
* 0.01≤p<0.05, ** 0.001≤p<0.01 & *** p<0.001

Table 5 Behavioural and housing characteristics & risk of *Aspergillus/Penicillium*

Dampness variables	Percent (n/d)	P value	Risk of fungus			
			Unadjusted		Adjusted∞	
			OR	95% (CI)	OR	95% (CI)
Homes built after 1965; no	35 (48/136)	0.36	Ref		Ref	
Yes	41 (38/92)		1.37	0.69-2.72	1.54	0.84-2.83
Type of house surveyed;						
Bungalow & flat	52 (44/85)	0.00	Ref		Ref	
House	29 (42/143)		0.39	0.22-0.68**	0.42	0.22-0.81*
Uses gas for heating; no	40 (47/117)	0.43	Ref		Ref	
Yes	35 (39/111)		0.78	0.41-1.46	0.86	0.46-1.63
Depth of home insulation;						
≥250	38 (39/103)	0.96	Ref		Ref	
<250	38 (47/123)		1.02	0.53-1.99	0.88	0.48-1.62
Double glazed windows with trickle vents; no	32 (20/63)	0.26	Ref		Ref	
Yes	40 (42/104)		1.48	0.73-3.02	1.76	0.71-4.34
Levels of deprivation, IMD;						
9-21 0 low deprivation	45 (40/89)	0.19	Ref		Ref	
≥21-26	32 (20/63)		0.54	0.25-1.21	0.55	0.25-1.23
≤26-60 high deprivation	34 (26/76)		0.60	0.29-1.27	0.60	0.30-1.22
Room with mechanical ventilation; no	32 (34/106)	0.08	Ref		Ref	
Yes	46 (28/61)		1.80	0.94-3.44	1.66	0.85-3.23
Windows open during survey; no	37 (24/65)	0.89	Ref		Ref	
Yes	38 (61/161)		1.05	0.52-2.13	1.12	0.57-2.17
Does not heat home due to cost; no	36 (60/169)	0.26	Ref		Ref	
Yes	44 (23/52)		1.47	0.69-3.16	1.34	0.66-2.72
Room carpeted; no	40 (40/101)	0.58	Ref		Ref	
Yes	36 (45/125)		0.78	0.40-1.50	0.76	0.40-1.45
Room decorated / fungicide used in last 12 months; no	38 (46/120)	0.82	Ref		Ref	
Yes	40 (30/75)		1.15	0.54-2.46	1.12	0.57-2.21
Room cluttered with furniture; no	35 (40/116)	0.12	Ref		Ref	
Yes	46 (36/79)		1.61	0.82-3.19	1.62	0.87-2.99
Room floor hovered; no	49 (34/70)	0.02	Ref		Ref	
Yes	31 (37/120)		0.44	0.21-0.91*	0.46	0.21-0.98*
Presence of pets; no	37 (43/116)	0.51	Ref		Ref	
Yes	42 (33/79)		1.21	0.60-2.42	1.22	0.64-2.32
Participant said they would benefit from receiving health information; no	32 (27/85)	0.18	Ref		Ref	
Yes	41 (51/125)		1.59	0.76-3.33	1.48	0.71-3.08

∞ Adjusted model for number of occupants, outdoor temperature and whether participants stated they ventilate to minimise damp / fungal growth
* 0.01≤p<0.05, ** 0.001≤p<0.01 & *** p<0.001

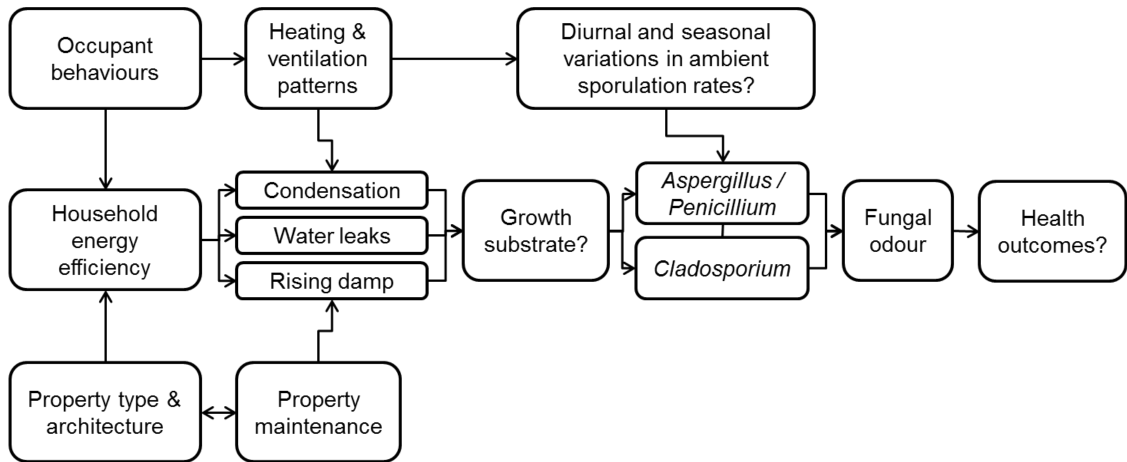
Table 6 Behavioural and housing characteristics & risk of *Cladosporium*

Dampness variables	Percent (n/d)	P value	Risk of fungus			
			Unadjusted		Adjusted∞	
			OR	95% (CI)	OR	95% (CI)
Homes built after 1965; no Yes	23 (31/136) 28 (26/92)	0.35	Ref 1.27	 0.49-3.26	Ref 1.05	 0.45-2.45
Type of house surveyed; Bungalow & flat House	 9 (8/85) 34 (49/143)	 0.00	 Ref 5.04	 2.08-12.26***	 Ref 3.56	 1.41-9.05**
Uses gas for heating; no Yes	27 (32/117) 23 (25/111)	0.40	Ref 0.73	 0.28-1.89	Ref 0.66	 0.29-1.50
Depth of home insulation; ≥250 <250	 25 (26/103) 25 (31/123)	 0.99	 Ref 1.22	 0.46-3.24	 Ref 1.40	 0.60-3.23
Double glazed windows with trickle vents; no Yes	 29 (18/63) 26 (27/104)	 0.71	 Ref 0.99	 0.37-2.62	 Ref 1.32	 0.47-3.70
Levels of deprivation, IMD; 9-21 0 low deprivation ≥21-26 ≤26-60 high deprivation	 27 (24/89) 27 (17/63) 21 (16/76)	 0.62	 Ref 0.89 0.76	 0.28-2.80 0.24-2.34	 Ref 0.89 0.86	 0.30-2.64 0.32-2.29
Room with mechanical ventilation; no Yes	 30 (32/106) 21 (13/61)	 0.21	 Ref 0.53	 0.21-1.35	 Ref 0.62	 0.25-1.54
Windows open during survey; no Yes	 20 (13/65) 27 (43/161)	 0.29	 Ref 1.56	 0.67-3.62	 Ref 1.53	 0.67-3.46
Does not heat home due to cost; no Yes	 27 (45/169) 21 (11/52)	 0.43	 Ref 0.91	 0.30-2.73	 Ref 1.00	 0.38-2.64
Room carpeted; no Yes	28 (28/101) 22 (28/125)	0.36	Ref 0.78	 0.37-1.65	Ref 0.70	 0.34-1.46
Room decorated / fungicide used in last 12 months; no Yes	 28 (33/120) 20 (15/75)	 0.24	 Ref 1.04	 0.34-3.20	 Ref 0.93	 0.34-2.55
Room cluttered with furniture; no Yes	 22 (25/116) 29 (23/79)	 0.23	 Ref 1.58	 0.68-3.68	 Ref 1.49	 0.67-3.33
Room floor hovered; no Yes	26 (18/70) 25 (30/120)	0.91	Ref 0.79	 0.25-2.51	Ref 0.51	 0.18-1.44
Presence of pets; no Yes	27 (31/116) 22 (17/79)	0.41	Ref 0.85	 0.35-2.09	Ref 0.87	 0.37-2.04
Participant said they would benefit from receiving health information; no Yes	 29 (25/85) 24 (30/125)	 0.38	 Ref 0.84	 0.34-2.11	 Ref 0.88	 0.38-2.02

∞ Adjusted model for number of occupants, outdoor temperature and whether participants stated they ventilate to minimise damp / fungal growth
* 0.01≤p<0.05, ** 0.001≤p<0.01 & *** p<0.001

Figure

Figure 1 Conceptual diagram of factors modifying fungal diversity



Supplementary Material
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