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1 **The seaweed fly (Coelopidae) can facilitate environmental survival and**
2 **transmission of *E. coli* O157 at sandy beaches**

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13

14 **ABSTRACT**

15 The sustainable management of recreational beaches is essential for minimising risk of human
16 exposure to microbial pathogens whilst simultaneously maintaining valuable ecosystem services.
17 Decaying seaweed on public beaches is gaining recognition as a substrate for microbial
18 contamination, and is a potentially significant reservoir for human pathogens in close proximity to
19 beach users. Closely associated with beds of decaying seaweed are dense populations of the
20 seaweed fly (Coelopidae), which could influence the spatio-temporal fate of seaweed-associated
21 human pathogens within beach environments. Replicated mesocosms containing seaweed
22 inoculated with a bioluminescent strain of the zoonotic pathogen *E. coli* O157:H7, were used to
23 determine the effects of two seaweed flies, *Coelopa frigida* and *C. pilipes*, on *E. coli* O157:H7 survival
24 dynamics. Multiple generations of seaweed flies and their larvae significantly enhanced persistence
25 of *E. coli* O157:H7 in simulated wrack habitats, demonstrating that both female and male *C. frigida*
26 flies are capable of transferring *E. coli* O157:H7 between individual wrack beds and into the sand.
27 Adult fly faeces can contain significant concentrations of *E. coli* O157:H7, which suggests they are
28 capable of acting as biological vectors and bridge hosts between wrack habitats and other seaweed
29 fly populations, and facilitate the persistence and dispersal of *E. coli* O157:H7 in sandy beach
30 environments. This study provides the first evidence that seaweed fly populations inhabiting natural
31 wrack beds contaminated with the human pathogen *E. coli* O157:H7 have the capacity to amplify the
32 hazard source, and therefore potential transmission risk, to beach users exposed to seaweed and
33 sand in the intertidal zone. The risk to public health from seaweed flies and decaying wrack beds is

34 usually limited by human avoidance behaviour; however, seaweed fly migration and nuisance inland
35 plagues in urban areas could increase human exposure routes beyond the beach environment.

36

37 **Key words:** biological vector; insect; public health; wrack; zoonotic pathogen

38

39

40 **HIGHLIGHTS**

41 Seaweed flies (Coelopidae) enhance survival of *E. coli* O157:H7 in seaweed and sand.

42 *C. frigida* flies vector *E. coli* O157:H7 to seaweed and sand.

43 *C. frigida* female flies ingest and excrete *E. coli* O157:H7 more rapidly than males.

44 Coelopidae are bridge hosts of *E. coli* O157:H7 in sandy beach environments.

45

46

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57

58 **1. INTRODUCTION**

59 Shiga-toxin (*stx*) producing *Escherichia coli* (STEC) serotype O157:H7 is often carried in the
60 digestive tracts of various animal reservoirs including cattle and other ruminants [Ferens and Hovde,
61 2011]. Human infection by *E. coli* O157:H7 can cause acute gastrointestinal illness, presenting
62 primarily in the form of diarrhoea, but can also cause haemolytic uremic syndrome (HUS) and lead to
63 permanent liver damage [Griffin and Karmali, 2017]. Importantly, infection can be caused by
64 extremely low infectious dose rates (< 10 - 50 viable cells), and can be fatal for young children or
65 those with compromised immune systems [Teunis et. al., 2004, Lim et. al., 2010]. There is also
66 growing concern about the multiple antimicrobial resistance of shiga toxin-producing *Escherichia*
67 *coli*, due in part to indiscriminate application of antibiotics to livestock and the various direct and
68 indirect pathways by which humans can become infected (Hoelzer et. al., 2017). Cattle, human,
69 environmental and food sources of 129 *E. coli* O157:H7 isolates have exhibited resistance to at least
70 five antimicrobials (Srinivasan et. al., 2007). Coupled with the increased risk of antibiotic dosing
71 provoking HUS in clinical patients (Freedman et. al., 2016), there is an important public health risk
72 posed by under-reported reservoirs and undocumented vectors of *E. coli* O157:H7 in the
73 environment. Human *E. coli* O157:H7 infection most commonly occurs through consumption of
74 contaminated food and water, person-to-person contact, or exposure to animal carriers [Kintz et. al.,
75 2017]. The epidemiology of *E. coli* O157:H7 is shaped by multiple routes of exposure throughout the
76 wider environment in which human-animal ecological niches overlap, which coupled with the
77 specific survival characteristics of *E. coli* O157:H7 in non-host habitats prevents accurate prediction
78 of the spatio-temporal fate of this pathogen in the environment [Chapman et. al., 2017, van Elsas et.
79 al., 2011]. Hence, our incomplete understanding of the survival capacity of *E. coli* O157:H7 in hostile
80 secondary environments, together with a lack of accurate quantification tools, hampers efforts to
81 manage its public health risk [Quilliam et al., 2011, Young, 2016].

82 The level of risk of human infection by a zoonotic pathogen such as *E. coli* O157:H7 is partly
83 determined by the prevalence of infection amongst disease reservoirs and secondary (bridge) hosts

84 [Lloyd- Smith et. al., 2009]. Important bridge hosts known to spread and transmit *E. coli* O157:H7
85 directly and indirectly to humans are synanthropic (e.g. houseflies) and non-synanthropic (e.g. fruit
86 flies) species of fly (Diptera) [Pace et. al., 2017, Janisiewicz et. al., 1999]. Fly larvae are typically
87 nutritionally dependent on bacteria in their diet, although destructive gut enzymes and antimicrobial
88 substances enable the larvae of some species to produce near-sterile faecal excretions [Mumcuoglu
89 et. al., 2001, Nayduch and Burrus, 2017]. The environment is the principal source of bacterial
90 contamination of adult flies, and often occurs via direct ingestion from a feeding surface or indirectly
91 during grooming [Nayduch and Burrus, 2017]. Thereafter, bacteria attached to the fly exoskeleton
92 may be passively transferred to other surfaces, including from hairs, legs and adhesive feet, or
93 deposited via regurgitation or faecal excretions if the bacteria are capable of surviving passage
94 through the digestive tract [Sasaki et. al., 2000, Graczyk et. al., 2001, Sukontason et. al., 2006]. *E. coli*
95 O157:H7 has been found to replicate on housefly mouthparts thus extending the duration of its
96 expression in fly faeces, and to grow on house fly exoskeletons and in vomit spots [Kobayashi et. al.,
97 1999, Wasala et. al., 2013]. The cumulative effect of these mechanical and biological interactions of
98 flies with pathogens is to enhance their capacity for disease transmission.

99 Recreational beach environments are vulnerable to downstream transport of human
100 pathogens, and virulence *stx*₂ genes of pathogenic *E. coli* have been isolated from swash zone sand
101 of freshwater beaches (Cho et. al., 2016, Bauer and Alm, 2012). The source of an outbreak of *E. coli*
102 O157:H7 infection amongst seven children playing on a UK marine beach, for example, was
103 identified as a contaminated stream draining an area of upstream cattle grazing, recently subjected
104 to heavy rainfall (Ihekweazu et. al., 2006). Although seawater and sand are known reservoirs of
105 faecal bacteria [Solo-Gabriele et al., 2016], additional reservoirs for microbial pathogens within
106 beach environments include decaying piles of seaweed (wrack), which can also enhance the
107 persistence of *E. coli* in adjacent seawater and sand [Imamura et. al., 2011, Quilliam et. al., 2014].
108 Stranded, decaying wrack is thus a potentially important reservoir for *E. coli* O157:H7 and can
109 concentrate human exposure risks within recreational spaces such as bathing water beaches. In

110 beach environments, the public often share their recreational space with seaweed flies (Coelopidae),
111 which are attracted to decaying wrack beds within a few hours of deposition along the strandline
112 [Dobson, 1974a]. Seaweed flies undergo their entire life-cycle within wrack beds, and often form
113 dense populations. In northern Europe, the dominant species are *C. frigida* (Fabricius) and *C. pilipes*,
114 and detached seaweed induces both male mating behaviour and female ovipositioning, with *C.*
115 *frigida* preferentially laying eggs on *Laminaria* spp. and *C. pilipes* favouring *Fucus* spp. [Dobson,
116 1974a, Edward et. al., 2007, Dunn et. al., 2002]. Although the potential for decaying wrack beds to
117 function as reservoirs of human pathogenic bacteria is gaining recognition [Quilliam et. al., 2014,
118 Russell et. al., 2014], there are no published studies addressing the risk of seaweed flies
119 disseminating human pathogens between wrack habitats.

120 Identification of all possible modes of direct and indirect transmission of human microbial
121 pathogens in the coastal zone will enable more effective management of the potential public health
122 risk in that environment [Young, 2016, Caron et. al., 2015]. Therefore, the aim of this study was to
123 establish whether *C. frigida* and *C. pilipes* can influence the survival and transmission dynamics of *E.*
124 *coli* O157:H7. Furthermore, the use of a chromosomally *lux*-marked (Tn5 *luxCDABE*) *E. coli* O157:H7
125 serotype (Ritchie et. al., 2003) provided the opportunity to measure bioluminescence of the
126 pathogen as a proxy for changes in its metabolic activity in decaying seaweed and in sand in the
127 presence of flies and larvae, and in response to ingestion by both life stages. Specifically, the
128 objectives were to determine whether the presence and feeding activity of multiple generations of
129 flies and larvae respectively and of both species had consequences for the persistence and metabolic
130 activity of *E. coli* O157:H7 on decaying seaweed and in beach sand; determine the effect of *C. frigida*
131 larval feeding, developmental stage and larval-associated native microbiota, and the competitive
132 effect of natural wrack bed bacterial communities, on the survival and metabolic activity of *E. coli*
133 O157:H7 in the larval gut, on decaying seaweed and in beach sand; establish the capacity for *C.*
134 *frigida* flies to transmit, and function as bridge hosts of, *E. coli* O157:H7, investigate whether vector
135 competence differed between females and males, and determine the metabolic activity of the

136 vectored pathogen, and finally to quantify the contribution of faecal excretion of metabolically
137 active *E. coli* O157:H7 to transmission by *C. frigida* adults following pathogen ingestion, and identify
138 whether capacity for biological transmission differed between females of different reproductive
139 stage and males. It was hypothesised that (i) the presence of seaweed flies and larvae facilitates the
140 persistence and activity of *E. coli* O157:H7 in wrack beds and underlying sand; (ii) larval feeding
141 suppresses *E. coli* O157:H7 populations and activity in their seaweed substrate by inactivating the
142 pathogen during larval digestion, that this mode of action is mediated both by larval developmental
143 stage and the presence of native gut and exoskeleton bacteria, and that natural bacterial
144 assemblages in wrack beds limit *E. coli* O157:H7 growth through competition; (iii) *C. frigida* flies,
145 particularly females, are a bridge host and transmission pathway for metabolically active *E. coli*
146 O157:H7, and (iv) metabolically active *E. coli* O157:H7 can be dispersed and survive in the
147 environment via biological transmission in faecal excretions, females exhibit a greater capacity for
148 this mode of transmission than do males, and females with developing eggs imbibe more liquid than
149 females with mature eggs.

150

151 **2. METHODS**

152 **2.1 Preparation of Coelopidae colonies.**

153 Colonies of *C. frigida* and *C. pilipes* were cultured from wild larvae collected from stranded
154 wrack beds on an exposed and natural sandy beach in Fife, Scotland (56°11.191'N, 2°48.679'W).
155 Larvae were grown in a controlled environment cabinet (Reftech B.V., Netherlands) at 25 °C ± 2 °C, a
156 relative humidity of 60 % and a photoperiod of 12 h, and fed with fresh, finely minced (0.5 cm²)
157 seaweed species characteristic of a stranded wrack bed: (*Laminaria digitata* (Hudson) (40 %),
158 *Laminaria hyperborea* (Gunnerus) (20 %), *Fucus serratus* (L.) (20 %), *Ascophyllum nodosum* (L.) (10
159 %), *Saccharina latissima* (L.) (5 %), *Palmaria palmata* (L.) (3 %) and *Rhodomela confervoides* (Hudson)
160 (2 %). Newly emerged adults were collected as virgins twice daily through attraction to a light box.
161 Following 10 s anaesthesia with CO₂, flies were classified by species and sex, and stored at 4 °C in

162 ventilated 150 ml plastic Erlenmeyer flasks containing cotton wool soaked in a 50 % glucose solution;
163 all flies were used in experimental mesocosms within 96 h.

164

165 **2.2 . Experimental design.**

166 A total of four experiments were conducted. Three utilised mesocosms containing multiple
167 individuals designed to investigate Coelopidae population level interactions with *E. coli* O157:H7 in
168 simulated wrack bed habitat comprising decaying seaweed and underlying sand. In the first study, (i)
169 *C. frigida* and *C. pilipes* flies were introduced to mesocosms to determine the effect of mixed species
170 colonies (and multiple generations of flies and larvae) on *E. coli* O157:H7 persistence and activity in
171 wrack bed habitat over several months. The second mesocosm experiment (ii) sought to examine
172 the effect of *C. frigida* larval feeding and development on *E. coli* O157:H7 persistence in simulated
173 wrack bed habitat, the facilitatory role of the larvae's native exoskeleton and gut microflora on their
174 capacity to digest the pathogen, and the competitive effect of natural wrack bed bacterial
175 communities on *E. coli* O157:H7. The third mesocosm study (iii) was designed to investigate whether
176 *C. frigida* flies were capable of transmitting *E. coli* O157:H7 between wrack bed habitats. A fourth
177 experiment (iv) employing microcosms containing single adult individuals fed known concentrations
178 of *E. coli* O157:H7 was intended to quantify at fine scale the role of biological transmission of the
179 pathogen by the flies to their vectoring capability.

180

181 2.3 Materials for experimental mesocosms.

182 Seaweed, sand and seawater were collected at low tide the day before starting each experiment.
183 Recently deposited seaweed (*Laminaria* spp. (70 %) and *Fucus* spp. (30 %)), was gathered from the
184 strandline; sand was collected from above the drift line and seawater from the surf zone. All
185 environmental materials were stored at 4 °C prior to transfer to mesocosms. Background *E. coli* and
186 total heterotrophic bacteria (THB) were enumerated in all seaweed, sand and seawater used in
187 experimental mesocosms, and pH and water content measured in the seaweed and sand (Table 1).

188 To quantify background *E. coli* and THB concentrations, four replicate samples of 10 g of seaweed or
 189 5 g of sand were added to 10 ml or 5 ml of sterile seawater, respectively, and then vortexed for 1
 190 minute. The supernatant was subsequently serially diluted with sterile seawater and 50 µl streaked
 191 onto Membrane Lactose Glucuronide Agar (MLGA) (CM1031, Oxoid) to enumerate presumptive *E.*
 192 *coli*, or R2A agar (CM0906, Oxoid) to enumerate THB. Seawater samples ($n = 4$) were shaken and 100
 193 ml vacuum-filtrated through a 0.45 µm cellulose acetate membrane (Microsart CN-filter, Sartorius
 194 Stedim Biotech GmbH, Goettingen, Germany) and transferred onto MLGA. Plates for *E. coli* were
 195 incubated at 37 °C for 24 h and plates for THB incubated at 18 °C for 48 h. For sand and seaweed
 196 samples, bacterial concentrations were expressed as CFU (colony forming units) g⁻¹ dry matter
 197 content (where representative seaweed and sand samples were dried at 80 °C for 24 h), or
 198 expressed as CFU 100 ml⁻¹ for sea water samples.

199

Experiment	Environmental parameter	Seaweed	Sand
1*	Water content (%)	74 ± 1	14 ± 0.2
	pH	-	9.6 ± 0.1
	<i>E. coli</i> (CFU g ⁻¹)	0	0
	Total heterotrophic bacteria (CFU g ⁻¹)	33 x 10 ³ (± 0.23)	52 ± 0.44
2 and 3 ⁺	Water content (%)	81 ± 0.2	12 ± 0.4
	pH	-	8.0 ± 0.2
	<i>E. coli</i> (CFU g ⁻¹)	< 10	0
	Total heterotrophic bacteria (CFU g ⁻¹)	110 x 10 ⁴ (± 0.36)	20 ± 0.13

200 * *E. coli* O157:H7 persistence

201 + *E. coli* O157:H7 survival during larval development and *E. coli* O157:H7 transmission by flies

202

203 **Table 1.** Characteristics of seaweed and sand used in mesocosm experiments. Values represent the
 204 means ± SE.

205

206 A non-toxigenic, chromosomally *lux*-marked (Tn5 *luxCDABE*) *E. coli* O157:H7 serotype
 207 [Ritchie et. al. 2003] was grown on Sorbitol MacConkey Agar (SMAC) (CM0813, Oxoid) supplemented

208 with cefixime and potassium tellurite (CT) (SR0172, Oxoid) at 37 °C for 24 h for the selective isolation
209 of *E. coli* O157:H7. Presumptive colonies of *E. coli* O157: H7 were confirmed by a latex agglutination
210 test (DR0260, Oxoid), and then grown in Luria-Bertani (LB) broth (CM1018, Oxoid) at 37 °C, at 100
211 rev min⁻¹, for 18 h. Cells were washed three times in phosphate buffered saline (PBS) and re-
212 suspended in PBS in preparation for use in experimental mesocosms. The bioluminescence
213 phenotype of the *lux* biomarker of this strain of *E. coli* O157:H7 is dependent on the cellular energy
214 status. As cellular metabolism requires energy, bioluminescence output can be used as a proxy for
215 the metabolic activity of the population of cells, and thus, bioluminescence allows a quick *in situ*
216 estimation of how metabolically active the *E. coli* O157:H7 population is [Quilliam et al., 2012].

217

218 **2.4 Persistence and activity of *E. coli* O157:H7 in the presence of Coelopidae colonies.**

219 This mesocosm experiment was designed to quantify the effect of the presence of multiple
220 generations of *C. frigida* and *C.pilipes* flies and larvae on *E. coli* O157:H7 persistence and metabolic
221 activity in seaweed and sand. A total of 8 mesocosms were established, each consisting of a 5 L
222 plastic container (Addis Ltd., UK) with paper towelling secured over a hole (10 cm x 10 cm) in the lid
223 to allow gas exchange. The mesocosms contained the following treatments: (A) both *E. coli* O157:H7
224 and flies present; (B) just *E. coli* O157:H7 with no flies.. Each treatment consisted of four replicate
225 mesocosms, and each mesocosm contained 1 kg of finely minced (0.5 cm²) seaweed (approximately
226 5- 6 cm depth) laid over 2 kg of sand (approximately 3 cm depth). For mesocosms containing *E. coli*
227 O157:H7, the inoculant was mixed with seaweed for 5 min in a stomacher bag with 200 ml of
228 seawater contaminated with *E. coli* O157:H7 (1.84×10^9 CFU ml⁻¹). Seaweed added to the control
229 mesocosms (those not containing *E. coli* O157:H7) was mixed with 200 ml of uncontaminated,
230 unsterilised seawater. To each mesocosm containing flies, 10 male and 10 female individuals of
231 both *C. frigida* and *C. pilipes* ($n = 40$) were added. All mesocosms were transferred to a controlled
232 environment cabinet and maintained at 20 °C ± 2 °C, with a relative humidity of 60 % and a
233 photoperiod of 12 h.

234 To enumerate bacterial concentrations from each replicate mesocosm, 10 g of seaweed or 5
235 g of sand were added to 10 ml or 5 ml of sterile seawater, respectively, and then vortexed for 1
236 minute. Luminescence (relative light units (RLU)) of the seaweed or sand supernatant was
237 immediately measured using a SystemSURE 18172 luminometer (Hygiena Int., Watford, UK) to
238 quantify relative *E. coli* O157:H7 metabolic activity. The remaining supernatant was serially diluted
239 using sterile seawater, plated onto either CT-SMAC or R2A plates, and incubated as described above.
240 *E. coli* O157:H7 and THB in both seaweed and sand were measured in each replicate mesocosm on
241 days 1, 2, 3, 4, 5, 6, 7, 8, 9, 11, 15, 23, 31, 43 and 56; mesocosms containing the flies were not
242 sampled on days 1 and 2 in order to allow mating to occur.

243

244 **2.5 Survival and activity of *E. coli* O157: H7 in *C. frigida* larvae and wrack habitat during larval
245 development.**

246 This mesocosm experiment aimed to quantify the influence of larval feeding and
247 development on the persistence and activity of *E. coli* O157:H7 associated with larvae and their
248 wrack habitat, and to examine the effect of the removal of the naturally occurring larval-associated
249 microbiota on the ability of larvae to digest or inactivate *E. coli* O157:H7. Mesocosms ($n = 160$)
250 consisted of 100 ml sterile plastic pots (Gosselin™, Fisher Scientific UK Ltd) containing 25 g of
251 seaweed (approximately 4 cm depth) placed on 20 g of sand (approximately 1.5 cm), with lids
252 comprised of paper towelling to allow gas exchange. Prior to placement within each treatment and
253 control mesocosm, the seaweed was divided into 1 kg batches, and inoculated by homogenising it
254 for 5 min in a stomacher bag with 200 ml of *E. coli* O157:H7 contaminated seawater (4.42×10^7 CFU
255 ml^{-1}). A pair of unmated *C. frigida* female and male flies was introduced to each mesocosm and
256 removed 4 days later. On day 5, the resulting larvae were removed from these mesocosms and
257 treated in one of four ways, (i) untreated, (ii) surface sterilised, (iii) starved, or (iv) surface sterilised
258 then starved. Untreated larvae and larvae subjected to surface sterilisation only were removed for 3
259 h before returning 10 to each mesocosm. Starvation involved moving larvae to an empty sterile

260 container for 24 h, before returning 10 to each mesocosm. Thus sampling of mesocosms containing
261 starved larvae lagged behind other treatments and the controls by 24 h. The aim of surface
262 sterilisation was to reduce the microbial communities on the larval surface (although not completely
263 eliminate them) and involved immersion in a 19:1 PBS:Ethanol solution for 1 min followed by two
264 rinses in sterile PBS. Weak disinfectant was used in place of a potentially more effective stronger
265 concentration in order to avoid incidental gut sterilisation of larvae due to larval ingestion of the
266 disinfectant during immersion.

267 There were two control treatments from which larvae were absent: 40 mesocosms
268 contained seaweed inoculated with *E. coli* O157:H7 laid on top of sand, and 40 mesocosms
269 contained seaweed and sand both pre-sterilised by autoclaving (121 °C for 15 mins), after which the
270 seaweed was inoculated with *E. coli* O157:H7. Destructive sampling of 10 larvae, 10 g seaweed and 5
271 g sand from replicate mesocosms ($n = 4$) from treatments and controls began six days after initial
272 inoculation of seaweed and continued for eight successive days. Larvae (and pupae) were handled
273 with sterile forceps and ground in 2 ml PBS in a 1.5 ml Eppendorf tube for 30 s with a micro pestle
274 (Anachem Ltd., Bedfordshire, UK). Luminescence of the supernatant was immediately measured,
275 and the homogenate serially diluted and plated onto either CT-SMAC or R2 agar as described above.
276 *E. coli* O157:H7 concentration and relative activity and THB concentration were also enumerated in
277 both seaweed and sand samples as described above. Mesocosms were maintained at 25°C ± 2°C, a
278 relative humidity of 60% and a photoperiod of 12 h.

279

280 **2.6 Transmission of *E. coli* O157:H7 by female and male *C. frigida* flies.**

281 Using mesocosms, vector competence of *C. frigida* flies for metabolically active *E. coli*
282 O157:H7 was assessed by investigating the capacity of females and males to separately contaminate
283 previously uncontaminated seaweed and sand. Mesocosms ($n = 80$) consisted of 100 ml sterile
284 plastic pots (Gosselin™, Fisher Scientific UK Ltd) containing 25 g of seaweed (approximately 4 cm)
285 placed on 20 g of sand (approximately 1.5 cm), with lids comprised of paper towelling. Prior to

placement within each mesocosm, the seaweed was divided into 1 kg batches, and inoculated with 200 ml of *E. coli* O157:H7 contaminated seawater (6.9×10^7 CFU ml⁻¹). Ten *C. frigida* flies were added to each mesocosm; 40 mesocosms contained female flies, and 40 mesocosms contained male flies. After 24 h, all female and male flies were moved to 80 new mesocosms that contained 25 g of uncontaminated seaweed (approximately 4 cm), placed on 20 g of sand (approximately 1.5 cm), with paper towelling lids. After a further 24 h, eight replicate mesocosms (four female, four male) containing transplanted flies were destructively sampled, with 10 flies, 10 g seaweed and 5 g sand sampled from each mesocosm on nine successive days. Each fly was anaesthetised by 10 s exposure to CO₂ gas, and ground in 2 ml PBS in a 1.5 ml Eppendorf tube for 30 s with a micro- pestle. Luminescence of each fly supernatant was immediately measured, and the concentrations of *E. coli* O157:H7 and THB in the remaining supernatant determined as described above. *E. coli* O157:H7 and THB were enumerated, and relative activity measured, in the seaweed and sand as described above. All mesocosms were maintained at 25 °C ± 2 °C, at a relative humidity of 60 % and a photoperiod of 12 h.

300

301 **2.7 Contribution of faecal excretion to transmission of *E. coli* O157:H7 by female and male *C.***
302 ***frigida* flies..**

303 The potential for adult *C. frigida* faeces to facilitate the transmission and survival of *E. coli* O157:H7 was determined in mated females ($n = 240$), virgin females ($n = 240$) and virgin males ($n = 240$) in microcosms taking the form of individually enclosed Petri dishes (diameter 55 mm). Females were mated two days prior to the beginning of the experiment, and flies for all treatments were starved for 24 h before sampling began. Each Petri dish contained a sterile Eppendorf tube lid containing a feeding solution made from the liquid from decaying *L. digitata*. Half of the Petri dishes for each treatment ($n = 120$) contained feeding solution that had been contaminated by 250 µl of *E. coli* O157:H7 (1.09×10^2 CFU µl⁻¹), whilst the control groups ($n = 120$) received 250 µl of feeding solution uncontaminated with *E. coli* O157:H7. Previous observations determined that most

312 seaweed flies typically began producing faecal droplets 6 h after introduction of the feeding solution,
313 and that fly mortality began after 24 h. Thus, the fly from each experimental and control mesocosm
314 ($n = 40$ for each treatment) were sampled at 6 h, 12 h and 24 h, and *E. coli* O157:H7 concentration
315 and relative activity measured in each individual fly, and in fly faeces. Faecal droplets belonging to
316 each individual fly were counted, and a sterile toothpick used to transfer faeces from individual flies
317 to 5 ml of LB Broth. Faeces were enriched overnight for 18 h at 100 rpm at 37 °C; cells were
318 centrifuged, washed three times and re-suspended in PBS. Luminescence was quantified, and the
319 solution serially diluted and plated onto CT-SMAC media to enumerate *E. coli* O157:H7
320 concentrations. Petri dish microcosms were maintained at 25 °C ± 2 °C, at a relative humidity of 60 %
321 and a photoperiod of 12 h.

322

323 **2.8 Statistical analysis**

324 Data were normally distributed following \log_{10} transformation, and analysis of variance
325 (ANOVA) was applied to the data (SPSS 24.0 software, SPSS Inc. Chicago, IL, USA). A repeated
326 measures (rm) ANOVA was used to test the effect of Coelopidae presence on *E. coli* O157:H7
327 concentration and relative activity in seaweed and sand, and a factorial ANOVA followed by Tukey
328 post-hoc tests used to analyse the effect of larval feeding on *E. coli* O157 concentration, vector
329 competency and the capacity of seaweed flies for biological transmission. Differences were
330 considered significant at the $P \leq 0.05$ level.

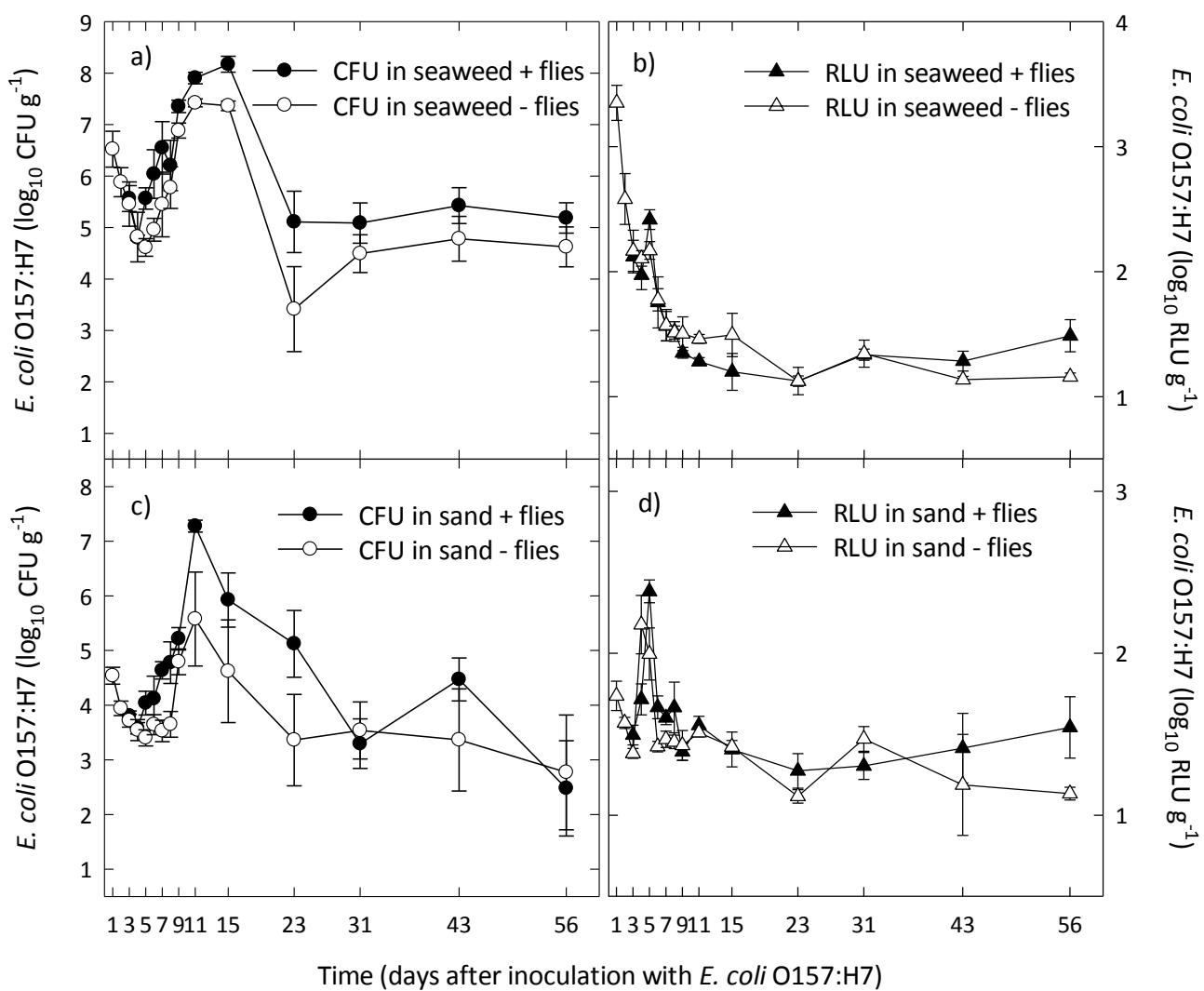
331

332 **3. RESULTS**

333 **3.1 Persistence and activity of *E. coli* O157:H7 in the presence of Coelopidae colonies.**

334 The presence of *C. frigida* and *C. pilipes* flies significantly enhanced survival of *E. coli*
335 O157:H7 attached to seaweed ($P < 0.001$) (Fig. 1a), and in the underlying sand ($P < 0.05$) (Fig. 1c),
336 compared to mesocosms where flies were absent. Regardless of the presence or absence of flies, the
337 concentration of *E. coli* O157:H7 peaked significantly in seaweed between days 9 and 15, and in sand

338 on day 11 ($P < 0.05$). Subsequent *E. coli* O157:H7 die-off to day 23 in seaweed was rapid in both
 339 treatments, reaching a concentration ~ 1 log CFU g $^{-1}$ lower in the absence of flies than in seaweed
 340 associated with flies ($P < 0.05$). *E. coli* O157:H7 levels in sand were ~ 1 log CFU g $^{-1}$ higher in the
 341 presence of flies than in the absence of flies between days 11 and 23 ($P < 0.05$), but the rate *E. coli*
 342 O157:H7 die-off in sand over two months was not significantly different between treatments. *E. coli*
 343 O157:H7 remained detectable in both seaweed and sand up to day 56. The presence of flies exerted
 344 no influence on the luminescence of *E. coli* O157:H7 in seaweed or sand (Fig. 1b and d).
 345



346
 347

348 **Fig. 1** Concentration in CFU (circles) in seaweed (a) and luminescence in RLU (triangles) in seaweed
349 (b) of *E. coli* O157:H7 in mesocosms containing either flies (filled symbols) or no flies (open symbols).
350 Concentration in CFU (circles) in sand (c) and luminescence in RLU (triangles) in sand (d) of *E. coli*
351 O157:H7 in mesocosms containing either flies (filled symbols) or no flies (open symbols). Each
352 mesocosm contained equal numbers of both *C. frigida* and *C. pilipes* flies. Data points represent the
353 means ± SE

354 **3.2 Survival and activity of *E. coli* O157: H7 in *C. frigida* larvae and wrack habitat during larval
355 development.**

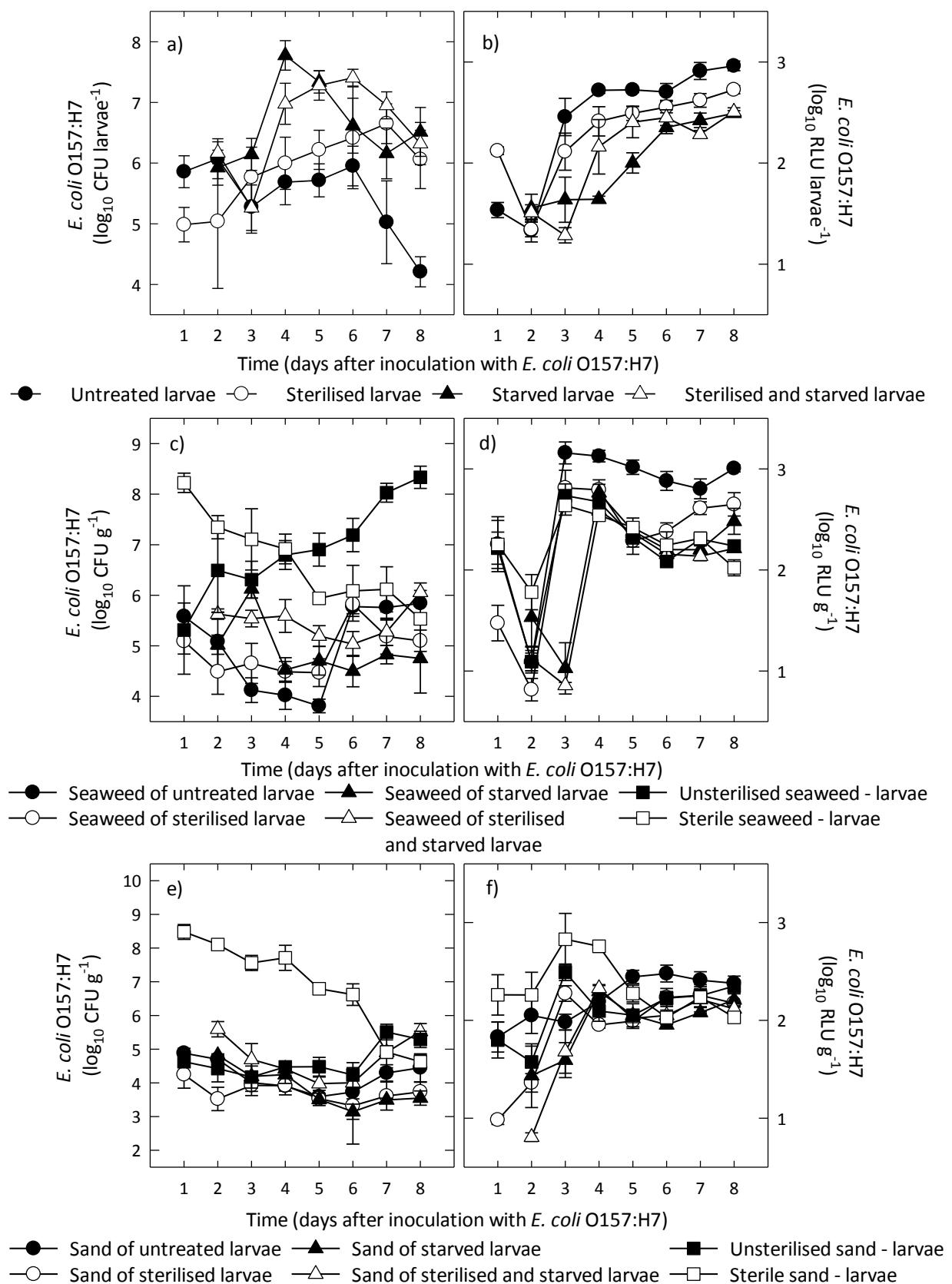
356 By day 7, *E. coli* O157:H7 concentrations associated with untreated larvae fell to ~1 log CFU
357 below that associated with sterilised, and starved and sterilised, larvae ($P < 0.05$), and by day 8 was
358 ~1.5 log CFU lower than levels detected in all treated larvae ($P < 0.05$) (Fig. 2a). Reduction of the gut
359 microbiota of larvae due to 24 h starvation led to significantly higher levels (~2 log CFU) of *E. coli*
360 O157:H7 associated with starved larvae compared with untreated and surface sterilised larvae on
361 day 4 ($P < 0.05$). Luminescence of *E. coli* O157:H7 increased significantly in untreated and sterilised
362 larvae between days 2 and 3, and in starved, and starved and sterilised larvae, between days 3 and 4
363 ($P < 0.001$) (Fig. 2b). Luminescence of *E. coli* O157:H7 associated with untreated larvae exceeded
364 that of all larvae that had reduced gut microbiota on days 3, 4, 7 and 8 ($P < 0.05$), and of starved
365 larvae on day 5 ($P < 0.05$).

366 The presence of larvae suppressed *E. coli* O157:H7 concentrations in seaweed, compared
367 with the non-sterile larvae-free mesocosms in which the concentration of *E. coli* O157:H7 associated
368 with the seaweed increased over 8 days to 2 – 3 log CFU g⁻¹ higher than all mesocosms containing
369 larvae ($P < 0.001$) (Fig. 2c). Concentrations of *E. coli* O157:H7 in seaweed associated with untreated
370 larvae increased rapidly by ~2 log CFU g⁻¹ between days 5 and 6 ($P < 0.05$), whereas no significant
371 change over time was observed in *E. coli* O157:H7 levels in seaweed associated with treated larvae.
372 The luminescence of *E. coli* O157:H7 associated with seaweed in the mesocosms containing larvae
373 and in the larvae-free mesocosms increased significantly between days 2 and 3 ($P < 0.05$), before
374 levelling off (Fig. 2d). Luminescence in seaweed in the mesocosms containing untreated larvae was

375 significantly higher from day 3 onwards ($P < 0.05$), than in seaweed associated with larvae where the
376 gut microbiota had been reduced. However, the absence of natural seaweed microflora, and the
377 absence of larvae did not affect *E. coli* O157:H7 luminescence in seaweed. *E. coli* O157:H7 levels in
378 sand were not influenced by the presence or absence of larvae (Fig. 2e); however, from day 7 the
379 concentration of *E. coli* O157:H7 in the sand of mesocosms that contained either larvae that had
380 been both sterilised and starved, or the non-sterile mesocosms that contained no larvae, were
381 significantly higher than in the sand of mesocosms that contained either the surface-sterilised larvae
382 or the starved larvae ($P < 0.05$). The luminescence of *E. coli* O157:H7 in sand associated with larvae
383 and in larvae-free controls increased significantly between day 2 and 3 ($P < 0.05$), although there
384 was no significant difference between mesocosms that contained larvae and those that contained
385 no larvae (Fig. 2f).

386 In the absence of larvae and natural seaweed microflora, *E. coli* O157:H7 in seaweed and
387 sand of the sterile control mesocosms significantly exceeded levels in seaweed and sand in all
388 treatments containing larvae and of the non-sterile control on day 1 ($P < 0.001$). By day 8,
389 subsequent die-off of *E. coli* O157:H7 in seaweed in the sterile control mesocosms resulted in the
390 concentration being significantly lower than that of seaweed in the non-sterile control mesocosm (P
391 < 0.001), whilst *E. coli* O157:H7 concentration in sand by day 8 was no different to that in any of the
392 treatment or non-sterile control mesocosms. Luminescence of *E. coli* O157:H7 in sand was
393 significantly enhanced by the absence of natural microflora in the sterile control compared with the
394 non-sterile control on days 1, 2 and 4 ($P < 0.05$).

395



396
397

398 **Fig. 2** Concentration in CFU (a, c and e) and luminescence in RLU (b, d and f) of *E. coli* O157:H7 in *C. frigida* larvae (a – b), seaweed (c - d) and sand (e - f) in mesocosms containing either untreated

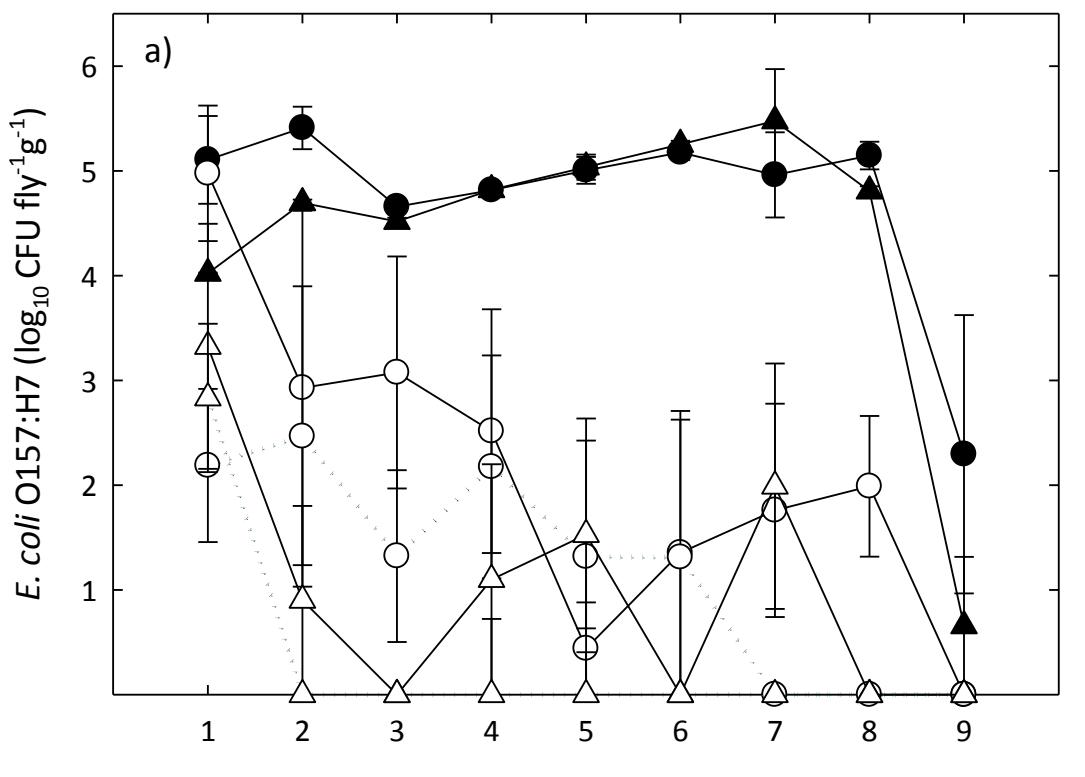
400 larvae (filled circles), surface sterilised larvae (open circles), starved larvae (filled triangles) or
401 sterilised and starved larvae (open triangles). Each mesocosm contained equal numbers of larvae.
402 Control mesocosms without larvae contained either unsterilized substrate (filled squares) or
403 sterilised substrate (open squares). Data points represent the means ± SE

404

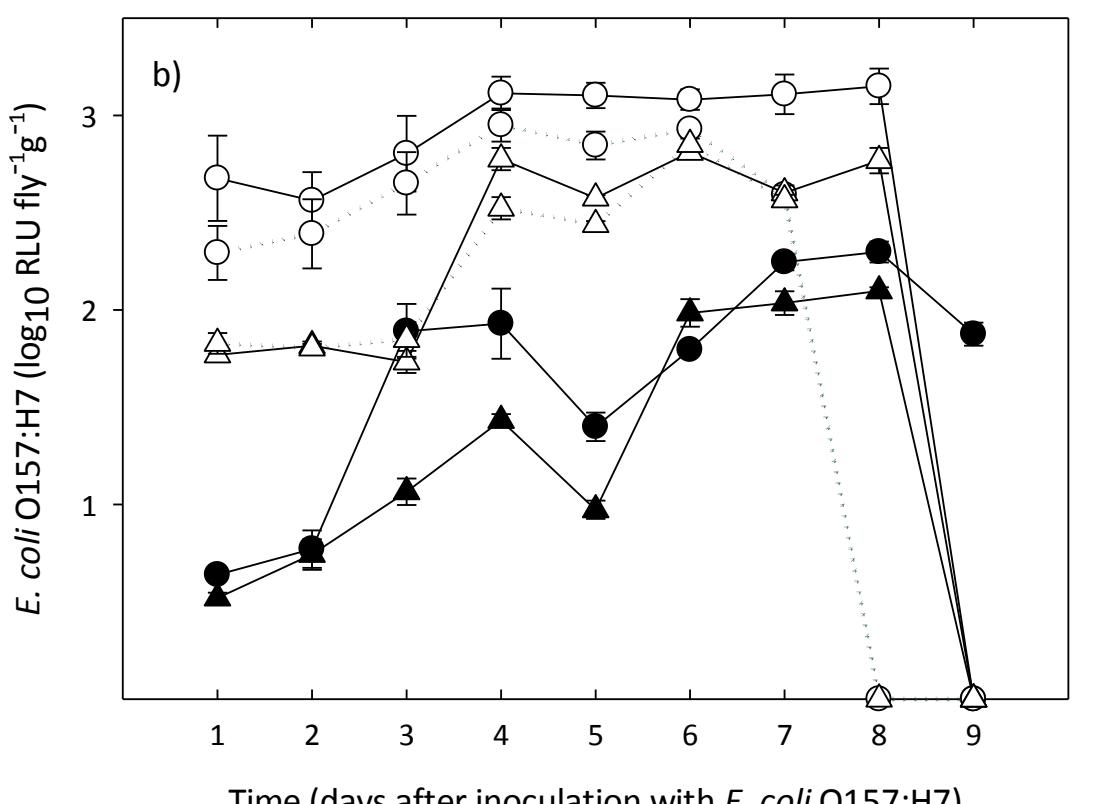
405 **3.3 Transmission of *E. coli* O157:H7 by female and male *C. frigida* flies.**

406 Female and male *C. frigida* flies transmitted *E. coli* O157:H7 from contaminated seaweed to
407 mesocosms previously free of the pathogen at concentrations > ~3 log CFU g⁻¹ to seaweed and > ~2
408 log CFU g⁻¹ to sand (Fig. 3a). The sex of the fly made no significant difference to the concentration of
409 *E. coli* O157:H7 associated with the flies following 24 h exposure to contaminated seaweed, or on
410 the subsequent persistence of the pathogen in flies until day 8. However, the gender of the flies in
411 the mesocosms significantly influenced *E. coli* O157:H7 concentrations in seaweed on day 3, at
412 which time pathogen levels on seaweed in female mesocosms were ~3 log CFU g⁻¹ higher than on
413 seaweed in male mesocosms ($P < 0.05$). *E. coli* O157:H7 concentrations in sand in female mesocosms
414 significantly exceeded that of sand in male mesocosms by ~2.5 log CFU g⁻¹ on day 2 ($P < 0.05$).
415 Luminescence of *E. coli* O157:H7 in female flies, and the seaweed and sand in their mesocosms, was
416 significantly higher than in male flies, seaweed and sand ($P < 0.001$) and increased significantly over
417 time in both female and male flies ($P < 0.001$) (Fig. 3b). Maximum levels of *E. coli* O157:H7
418 luminescence occurred in flies, seaweed and sand on days 8, 8 and 4 respectively in female
419 mesocosms and on days 8, 6 and 6 respectively in male mesocosms, significantly exceeding
420 luminescence levels recorded in flies, seaweed and sand at all preceding and subsequent sampling
421 points ($P < 0.05$).

422



● Female flies ○ Female sand ▲ Male seaweed
 ○ Female seaweed ▲ Male flies △ Male sand



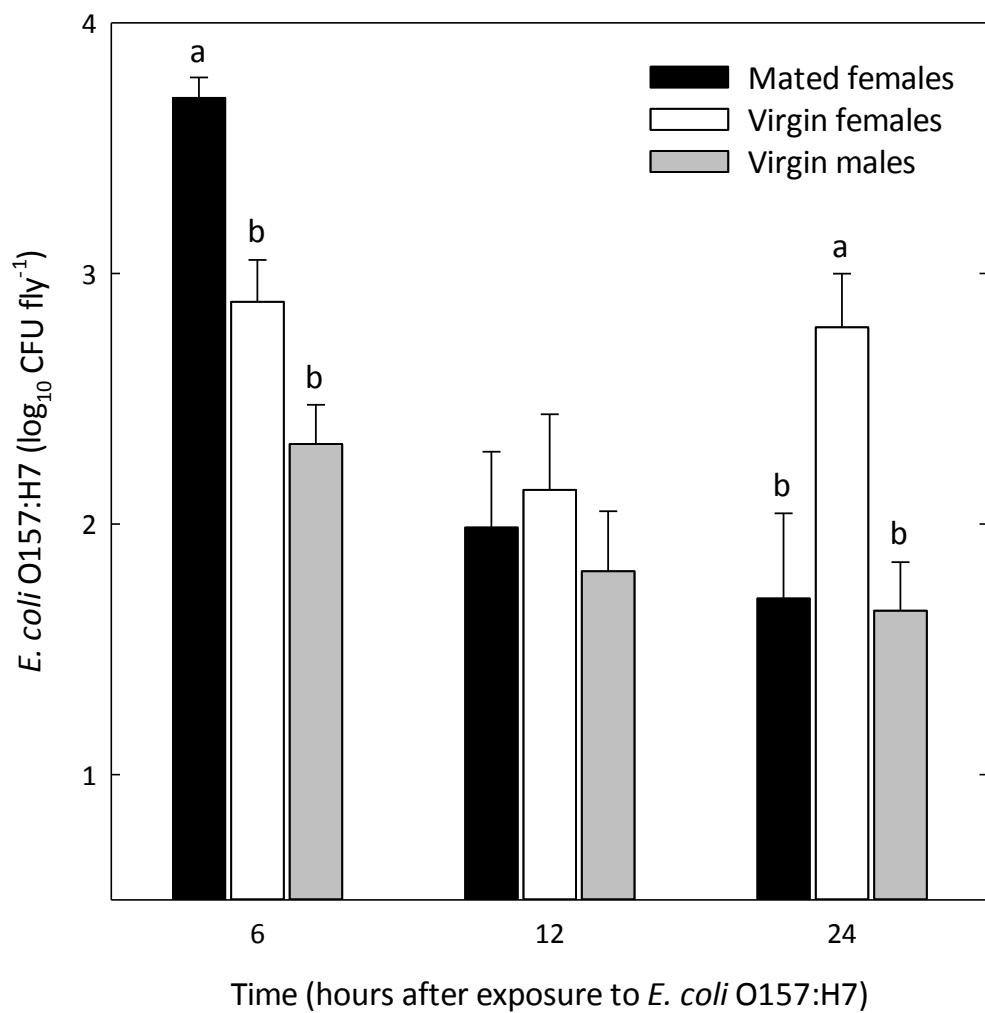
● Female flies ○ Female sand ▲ Male seaweed
 ○ Female seaweed ▲ Male flies △ Male sand

424 **Fig. 3** Concentration in CFU (a) and luminescence in RLU (b) of *E. coli* O157:H7 in female flies (filled
425 circles), male flies (filled triangles), seaweed (open circles) and sand (open circles with dotted line) in
426 female *C. frigida* mesocosms, and seaweed (open triangles) and sand (open triangles with dotted
427 line) in male *C. frigida* fly mesocosms. Data points represent the means ± SE

428 **3.4 Contribution of faecal excretion to transmission of *E. coli* O157:H7 by female and male *C.***
429 ***frigida* flies..**

430 After 6 h exposure to contaminated feeding solution, mated female flies were contaminated
431 with significantly (~ 1 log CFU fly $^{-1}$) more *E. coli* O157:H7 than either virgin females or males ($P < 0.05$)
432 (Fig. 4). By 12 h *E. coli* O157:H7 contamination had decreased in both females and males, with a
433 significant reduction in mated females between 6 h and 12 h ($P < 0.001$). After 24 h exposure to *E.*
434 *coli* O157:H7, 40 % of males, 7.5 % of mated females and 5 % of virgin females had died. The further
435 decrease in contamination by *E. coli* O157:H7 between 12 h and 24 h was not significantly different
436 between mated females and virgin males. However, between 12 h and 24 h *E. coli* O157:H7
437 concentrations in virgin females increased by ~ 1 log CFU fly $^{-1}$, and the final concentration at 24 h was
438 significantly higher than levels associated with mated females and virgin males ($P < 0.05$). *E. coli*
439 O157:H7 luminescence did not change significantly in female or male flies over 24 h, and there was
440 no significant difference between mated and virgin females at 6 h, 12 h or 24 h; however, the
441 luminescence of *E. coli* O157:H7 associated with males was consistently lower than in either female
442 fly group ($P < 0.05$).

443



444

445 **Fig. 4** *E. coli* O157:H7 concentrations in mated female flies (black bars), virgin female flies (white
446 bars) and virgin male flies (grey bars) following exposure to inoculated feeding liquid. At each time
447 point, bars that do not share a letter are significantly different from each other (two-way ANOVA, $P <$
448 0.05; Tukey's test, $P < 0.05$). Values represent the means +SE

449

450 Ingestion of *E. coli* O157:H7 had no effect on excretion levels in mated females, virgin
451 females or virgin males relative to controls, and the number of faecal excretions produced by all flies
452 in all mesocosms significantly increased between 6 h and 12 h, and again between 12 h and 24 h ($P <$
453 0.05). Faecal biomass and excretion rate were unaffected by the extent of egg maturation in
454 females, but significantly exceeded that of males over the entire 24 h period ($P < 0.05$). The *E. coli*
455 O157:H7 load in fly faeces increased significantly between 6 h and 12 h in females and males by ~2-

456 log CFU fly⁻¹ ($P < 0.001$) followed by a significant reduction of 3 - 4 log CFU fly⁻¹ in females and 2- log
457 CFU fly⁻¹ in males ($P > 0.001$) by 24 h (Fig. 5). The sex of the fly affected the concentration of *E. coli*
458 O157:H7 in faecal excretions, with females producing ~4 log CFU fly⁻¹ more of the pathogen in their
459 faeces than males at 6 h and 12 h, and ~2 - 3 log CFU fly⁻¹ more than males at 24 h ($P < 0.001$). The
460 extent of egg maturation in females also affected levels of the pathogen in female faeces after 24 h
461 exposure, with concentrations in mated females being ~1 log CFU fly⁻¹ greater than in virgin females
462 ($P < 0.05$). Luminescence of *E. coli* O157:H7 in the faeces of both female and male flies peaked at 12
463 h, increasing significantly between 6h and 12 h in male faeces and falling significantly between 12 h
464 and 24 h in virgin female faeces ($P < 0.05$). However, luminescence of *E. coli* O157:H7 in faeces did
465 not differ significantly between mated and virgin females during the 24 h; luminescence in faeces
466 from male flies remained significantly lower by comparison at all sampling times ($P < 0.001$).

467

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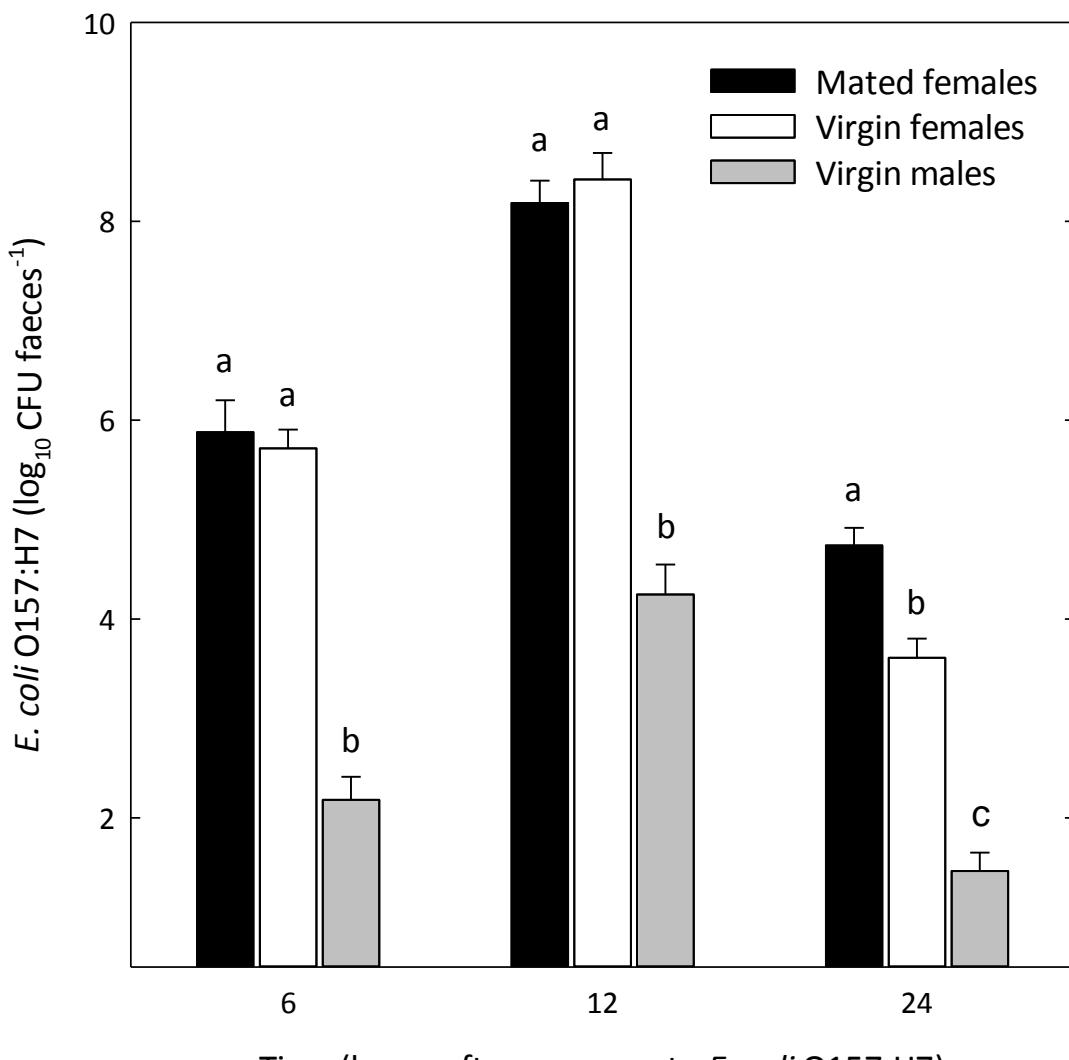


Fig. 5 E.

471 *coli* O157:H7 concentration in the faeces of mated female flies (black bars), virgin female flies
472 (white bars) and virgin male flies (grey bars) following exposure to inoculated feeding liquid. At each
473 time point, bars that do not share a letter are significantly different to each other (two-way ANOVA,
474 $P < 0.05$; Tukey's test, $P < 0.05$). Values represent the means +SE

475

476 4. DISCUSSION

477

478 The role of non-synanthropic Diptera in the environmental dissemination of human
479 pathogenic bacteria has not been previously examined within a public health context. This study has
480 demonstrated that an endemic species of seaweed fly (*C. frigida*) commonly found in dense
481 populations on public beaches throughout Europe is capable of facilitating the dispersal of *E. coli*

482 O157:H7 between individual seaweed habitats, and further transmission to beach sand. An
483 important mechanism for this transmission is in faecal excretions by adult flies. Furthermore, the
484 presence of both *C. frigida* and *C. pilipes* enhanced growth of *E. coli* O157:H7 in simulated wrack bed
485 environments, and in the underlying sand. Activity by multiple generations of flies and larvae in
486 decaying wrack beds modifies their habitat by altering the physio-chemical composition of the
487 substrate, and can facilitate microbial growth and persistence [Cullen et. al., 1987]. This study
488 provides the first evidence that seaweed fly populations inhabiting natural wrack beds contaminated
489 with the human pathogen *E. coli* O157:H7 have the capacity to amplify the hazard source, and
490 therefore potential transmission risk, to beach users exposed to seaweed and sand in the intertidal
491 zone.

492 Following ingestion of high concentrations of *E. coli* O157:H7, seaweed flies were rapidly
493 internally contaminated with the pathogen and within 6 h produced faeces containing viable
494 (metabolically active) *E. coli* O157:H7 bacteria at concentrations exceeding the infectious dose for
495 humans [Teunis et. al., 2004]. Homogenisation of whole flies in order to measure individual infection
496 with *E. coli* O157:H7 prevented evaluation of the separate contributions of bacterial attachment to
497 exoskeletons compared with ingestion to overall individual contamination. However, a degree of
498 external carriage of the pathogen was highly probable as flies of both sexes were observed in and on
499 feeding solution dishes. Therefore, greater carriage of *E. coli* O157:H7 after 6 h by mated females
500 than virgin females, and *vice versa* at 24 h, cannot be solely attributed to differences in ingestion
501 volume or rate, possibly due to stage of egg development [Sasaki et. al., 2000]. However, greater
502 contamination of mated female flies than males at 6 h, and of virgin females than males at 24 h may
503 indicate that reproductive biology influences ingestion volume and rate, most likely due to
504 physiological requirements associated with egg production.

505 A distinction should be drawn between studies in which fly exposure to a pathogen is via a
506 single food droplet, and those that allow constant feeding as in the present study, which more
507 realistically reflects the availability of the source in natural wrack beds. The former might be

508 expected to produce a steady decline over time in pathogen concentration in flies due to clearance
509 from their digestive tracts, whereas the latter may generate a more variable result due to multiple
510 feeding opportunities [Fleming et. al., 2014]. It is possible that the higher levels of activity of mated
511 and virgin female compared with males in the Petri dish microcosms may have resulted in additional
512 contamination of the female exoskeleton leading to higher overall *E. coli* O157:H7 concentrations on
513 mated females at 6 h and virgin females at 24 h. Importantly, the concentration of *E. coli* O157:H7
514 associated with the flies did not consistently increase during exposure to the contaminated feeding
515 solution; seaweed flies are therefore unlikely to be reservoirs of *E. coli* O157:H7, meaning that the
516 pathogen load associated with individuals is dependent upon levels of contamination in seaweed,
517 and is not influenced by disease maintenance amongst seaweed fly populations [Caron et. al., 2015].

518 The lower luminescence, and hence, relative metabolic activity, of *E. coli* O157:H7 associated
519 with male flies compared with females over 24 h may indicate that efficacy of inactivation of the
520 pathogen in the seaweed fly gut is partly related to the sex of the fly. This is unlikely however, given
521 that in Dipteran digestive tracts the efficacy of antibacterial effectors active against non-native gut
522 bacteria (the innate response) depends primarily on the species of fly and the vulnerability of the
523 bacterial species to that response [Nayduch and Burrus, 2017]. The fate of ingested *E. coli* O157:H7
524 may also be dose-dependent, meaning that below or above a certain dose threshold, bactericidal
525 substances in seaweed fly digestive tracts may be effective against ingested cells of the pathogen
526 [Kumar and Nayduch, 2016]. It is likely that external *E. coli* O157:H7 contamination of both mated
527 and virgin female seaweed flies will be greater than males due to their higher physical activity. By
528 contrast, if the majority of male contamination was internal and thus vulnerable to gut inactivation,
529 this might account for the consistently lower metabolic activity of *E. coli* O157:H7 associated with
530 male flies compared with females.

531 The mechanisms of bacterial transmission by flies to various surfaces via regurgitation and
532 faecal excretion are well established [Pava-Ripoll et. al., 2012], and passage of *E. coli* O157:H7
533 through the digestive tract of seaweed flies did not entirely inactivate this pathogen. Female *C.*

534 *frigida* produced more faecal excretions on average than male *C. frigida*, suggesting a more rapid
535 ingestion rate by females than males, which was also matched by a faster excretion rate. Clearance
536 of *E. coli* O157:H7 from the digestive tracts of female *C. frigida* was more rapid than their ingestion
537 rate, whereas males excreted *E. coli* O157:H7 at approximately the same rate as they ingested the
538 pathogen. The excretion rate by both female and male flies approximately doubled between 6 - 12
539 h, and 12 - 24 h, although this was not mirrored by the concentration of faecal *E. coli* O157:H7, most
540 likely due to the rate of pathogen die-off in the feeding solution. The concentration of *E. coli*
541 O157:H7 in the faeces of both sexes may be underestimated due to desiccation of most excreta by
542 12 h which would have affected recovery, and the decreasing availability of the feeding solution due
543 to evaporation over 24 h. However, these results do demonstrate that at 12 h after initial ingestion
544 of *E. coli* O157:H7, both female and male seaweed flies present the greatest risk of pathogen
545 transmission via faecal excretion.

546 Female and male seaweed flies were capable of vectoring *E. coli* O157:H7 to seaweed and
547 sand 24 hours after exposure to the pathogen. The faster rate of faecal production by female *C.*
548 *frigida*, and thus greater quantity of excretion of *E. coli* O157:H7 compared with males, represents
549 the underlying mechanism for the greater pathogen load transmitted by females than males to
550 simulated wrack habitats. Excretion droplets have been shown to be 'hotspots' of *E. coli* O157:H7
551 when the pathogen was fed to houseflies [Sasaki et. al., 2000], and viable populations of this
552 pathogen remained in seaweed fly faeces for at least 24 h after initiation of feeding on *E. coli*
553 O157:H7. The persistence of *E. coli* O157:H7 on seaweed and sand demonstrates that seaweed fly
554 excretions onto the surface of wrack and sand provided favourable conditions for *E. coli* O157:H7
555 persistence in these substrates. Survival of the pathogen in and on the flies is thus maintained by
556 continual ingestion and recontamination of the exoskeleton from the wrack habitat.

557 Temperature is a key determinant of the distribution of the cold-favouring *C. frigida* and a
558 northward shift in their northern European range in recent decades is a likely response, in part, to a
559 simultaneous warming trend in this region [Phillips et. al., 1995, Edward et. al., 2007, IPCC, 2013].

560 Mass migration of *C. frigida* adults over considerable distances has been reported, included nuisance
561 inland plagues in urban areas, possibly driven by sub-optimal habitat conditions, or alternatively
562 optimal conditions supporting high population densities [Egglishaw, 1961, Oldroyd, 1954]. The
563 phenomenon of inland emigration of seaweed flies indicates that the presence of decaying seaweed
564 is not a pre-requisite attractant for their dispersal, although the absence of wrack habitat inland
565 would prevent establishment of a population in that location. Female *C. frigida* can lay three
566 clutches of up to 80 eggs each and in mainland Europe this species is normally more abundant, and
567 experiences a faster egg to adult development time, than *C. pilipes* which lay single eggs [Dobson
568 1974a and 1974b, Edward et. al., 2007]. *C. frigida* larvae typically occur at densities of approximately
569 1000 larvae kg⁻¹ of seaweed, and in optimal conditions, *C. frigida* populations have the potential to
570 increase by approximately 200 times with each generation [Butlin et. al., 1984, Dobson, 1974a].
571 Thus, the potential for *E. coli* O157:H7 transmission by migrating female and male *C. frigida* within
572 and between beaches, and even inland, should not be underestimated. The ability of seaweed flies
573 to vector *E. coli* O157:H7 from contaminated wrack beds on beaches to recently deposited seaweed,
574 together with intraspecific transmission to other seaweed fly populations, therefore increases the
575 spatial reach of the risk of public exposure to this pathogen.

576 Persistence and growth of *E. coli* O157:H7 in seaweed and sand both in the presence and
577 absence of seaweed flies confirms that the simulated wrack environment facilitates long term
578 survival of *E. coli* O157:H7. Both seaweed and sand provide a source of environmental exposure to
579 the pathogen, which ensure that several generations of *C. frigida* and *C. pilipes* flies are continually
580 externally and internally contaminated and re-contaminated [Graczyk et. al., 2001]. Thus, a single
581 wrack bed could ensure the persistence of *E. coli* O157:H7 and subsequent vectoring by several
582 generations of seaweed flies; however, wrack beds in the natural environment are transient
583 habitats, often present for no more than a few days [Edward et. al., 2007]. Furthermore, laboratory
584 conditions protected *E. coli* O157:H7 from predation, UV radiation, and provided plentiful nutrients,
585 water and a favourable temperature [O'Mullan et. al., 2017]. Therefore, depending on vulnerability

586 to high tides and internal wrack bed temperatures attained, the observed growth of *E. coli* O157:H7
587 in seaweed and sand from day 4 may occur only sporadically in beach environments, meaning that
588 production of a single cohort of *E. coli* O157:H7 contaminated seaweed flies from a single wrack bed
589 is more likely than production of multiple cohorts. Additionally, the predominance of a single
590 bacterial species in the larval diets, and presence of two seaweed species only, contrasts with the
591 diverse microbial assemblage associated with the multiple seaweed species present in natural wrack
592 beds [Edward et. al., 2008]. Restriction to a sub-optimal diet, however, affected all treatments
593 equally, and seaweed fly larvae have been shown to survive on a monospecific diet of commensal *E.*
594 *coli*, suggesting that feeding and development were not greatly impaired by these experimental
595 conditions [Cullen et. al., 1987].

596 Interestingly, *C. frigida* adult flies facilitated the survival of *E. coli* O157:H7 in wrack bed
597 habitats over 56 days due to excretion of viable cells of the pathogen following ingestion, despite the
598 presence of multiple generations of larvae, whilst *C. frigida* larvae alone initially suppressed
599 populations of *E. coli* O157:H7 in the seaweed they inhabited. The onset of pupation on
600 approximately day 6 coincided with reductions in the concentration of *E. coli* O157:H7 in larvae and
601 pupae. This phenomenon, recorded for other species of fly larvae and human pathogens, could be
602 caused by cessation of feeding and subsequent voiding of digestive tracts prior to pupation, and the
603 destruction or inactivation of *E. coli* O157:H7 by gut microbes during metamorphosis [Lalander et.
604 al., 2013, Engel and Moran, 2013]. The efficiency by which these mechanisms reduced pathogen
605 loads in seaweed fly larvae, and resulted in increased *E. coli* O157:H7 on seaweed was dependent on
606 larvae possessing a full complement of native gut microbiota. Examination of the possible
607 contribution of loss of surface microbiota from seaweed fly larvae to reduction of *E. coli* O157:H7
608 concentrations within the larvae may have been confounded by ineffective surface disinfection of
609 larvae. This may have contributed to the lack of distinction between detected pathogen loads in
610 untreated and surface sterilised larvae throughout most of the sampling period. In the absence of
611 seaweed fly larvae and a diverse community of competing microbiota in seaweed and sand, the

612 initial rapid growth of *E. coli* O157:H7 was not sustained. In a contamination scenario of decaying
613 wrack beds contaminated with lower concentrations of *E. coli* O157:H7, seaweed fly larvae may be
614 capable of greater levels of pathogen reduction in seaweed and in the underlying sand. However,
615 concentrations of *E. coli* O157:H7 in or on larvae and pupae may still exceed that of their substrate
616 during their development, and at any level of contamination may be capable of passive transmission
617 of the pathogen between wrack habitats given that larvae washed by the sea from wrack beds can
618 survive 48 h of immersion in seawater [Dobson, 1974a]. Further research is required to determine if
619 *E. coli* O157:H7 can be transmitted trans-stadially between seaweed fly pupae and newly eclosed
620 adult flies, and at what concentration might *E. coli* O157:H7 ingested by larvae produce adults
621 immediately capable of vectoring the pathogen between wrack habitats [Schuster et. al., 2013].

622 The typical management response at popular recreational sandy beaches is to remove
623 decaying seaweed, which also appeals to the public's aesthetic preferences [Quilliam et al., 2015],
624 yet this has been shown to elicit either no change or an increase in faecal indicator organisms, such
625 as *E. coli*, in nearshore water [Russell et. al., 2014]. In addition, wrack removal reduces richness of
626 invertebrate species inhabiting wrack beds, including *C. frigida* and *C. pilipes* [Gilburn, 2012].
627 Management of diffuse and point sources of *E. coli* O157:H7 in the environment can help to reduce
628 *E. coli* O157:H7 inputs into beach environments, and farm-level strategies to reduce direct
629 defecation by livestock and diffuse agricultural runoff to the coastal zone are important to mitigate
630 the transfer of pathogens and nutrients to coastal environments [Young, 2016]. Excessive nitrogen
631 loading of coastal waters is a major cause of accelerated seaweed production, resulting in
632 unnaturally high levels of wrack biomass accumulating along coastlines [Anderson et. al., 2002]. In
633 such a scenario, and in combination with warmer temperatures as a result of climate change, the
634 availability of seaweed biomass for attachment by human pathogens including *E. coli* O157:H7,
635 combined with the subsequent growth of seaweed fly populations due to increased habitat
636 availability, could potentially increase the opportunity for seaweed flies to function as bridge hosts
637 and disseminate human pathogens at recreational beaches.

638

639

640 **CONCLUSION**

641 Seaweed flies and their larvae form large natural populations in recreational beach
642 environments and can act as bridge hosts of the human pathogen *E. coli* O157:H7. However, they
643 are restricted to decaying wrack beds and their dispersal is limited to beach environments where
644 that habitat occurs. Therefore, despite seaweed flies facilitating long-term survival of *E. coli* O157:H7
645 in seaweed and sand, and flies and larvae potentially disseminating the pathogen amongst individual
646 wrack beds and seaweed fly populations, both vectors and reservoirs are spatially constrained within
647 the environment. The risk to public health from seaweed flies and decaying wrack beds is usually
648 limited by human avoidance behaviour. However, beach sand can act as a significant reservoir with
649 which the public make far more deliberate contact, particularly following beach grooming and the
650 removal of seaweed.

651

652 **CONFLICT OF INTEREST**

653 Declarations of interest: none.

654

655 **COMPLIANCE WITH ETHICAL STANDARDS**

656 Ethical approval: All applicable international, national, and/or institutional guidelines for the care
657 and use of animals were followed. All procedures performed in studies involving animals were in
658 accordance with the ethical standards of the institution or practice at which the studies were
659 conducted. This article does not contain any studies with human participants performed by any of
660 the authors.

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