1 **Heliconiini butterflies can learn time-dependent reward associations**

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13

Abstract

15 For many pollinators, flowers provide predictable temporal schedules of resource availability,

16 meaning an ability to learn time-dependent information could be widely beneficial. However,

17 this ability has only been demonstrated in a handful of species. Observational studies of

18 *Heliconius* butterflies suggest that they may have an ability to form time-dependent foraging

19 preferences. *Heliconius* are unique among butterflies in actively collecting and digesting pollen,

20 a dietary behaviour linked to spatiotemporally faithful 'trap-line' foraging. Time-dependency of

21 foraging preferences is hypothesised to allow *Heliconius* to exploit temporal predictability in

22 alternative pollen resources, as well as contributing to optimal use of learnt foraging routes.

23 Here, we provide the first experimental evidence in support of this hypothesis, demonstrating

24 that *Heliconius hecale* can learn opposing colour preferences in two time periods. This shift in

25 preference is robust to the order of presentation, suggesting that preference is tied to the time of

26 day and not due to ordinal learning. However, we also show that this ability is not limited to

27 *Heliconius*, as previously hypothesised, but is also present in a related genus of non-pollen

28 feeding butterflies. This demonstrates that time learning pre-dates the origin of pollen-feeding

29 and may be prevalent across butterflies with less specialized foraging behaviours.

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31   **Keywords:** contextual learning, circadian memory, cognitive ecology, foraging, Lepidoptera,

32   pollinator.

1. Introduction

34

35   The specific cues providing animals with reliable information about resource availability vary

36   across environments, and this variation shapes which cues animals learn to use [1,2]. For

37   example, animals inhabiting dark environments may preferentially learn associations with

38   olfactory cues over visual cues [3,4]. These learning propensities can also evolve in response to

39   changes in conditions. In experimental conditions where colour cues are reliable, but odours are

40   not, *Drosophila* evolve strong visual learning propensities [5]. An animal's learning abilities are

41   therefore influenced by the relative reliability of the cues associated with key resources [6].

42   However, the context in which a cue is encountered can also have a role in shaping the reliability

43   of that cue [7,8].

44   For many pollinators, foraging for flowers occurs in the context of temporal variation in

45   resource profitability. Flowers tend to vary predictably in the availability of pollen and nectar

46   [9]. Consequently, some specialist nectarivores use time as a contextual cue to modulate their

47   foraging strategy [9]. For example, honey bees can consistently change their preference towards

48   particular visual cues throughout the day [10,11], and some nectarivorous ants remember the

49   time of day at which resources are most profitable [12,13]. However, the ability to learn time-

50   dependent associations has only been formally demonstrated in a handful of invertebrates,

51   including *Drosophila*, bees, and ants [11,14–21], and there is evidence that this ability can vary

52   across species from the same family [12,13,22]. Hence, the prevalence of this ability, and the

53   ecological factors that may account for its variability, are unclear.

54   *Heliconius* butterflies display a foraging behaviour not seen in the other 17,000+

55   described species of butterflies [23–25]. *Heliconius* actively collect and feed on pollen grains

56   from a restricted range of plants that occur in low densities, but flower continuously [23,26].

57   This dietary adaptation provides an adult source of essential amino acids [24], which is plausibly

58   linked to dramatically increased lifespan and reproductive longevity [27]. Pollen feeding is

59   associated with a suite of derived foraging and cognitive adaptations not seen in other tropical

60   butterflies, including fidelity to a local home-range [26,28], as well as temporally and spatially

61   faithful 'trap-lining' behaviour, in which individual butterflies consistently visit particular

62   flowers at specific times of day [26]. Foraging efficiency is reported to increase with experience,

63   suggesting trap-lines are learnt and refined throughout an individual's life [29]. Recent data also

64   suggest wild *Heliconius* visit particular flower species in a manner that coincides with the

65   maximal temporal availability of nectar and pollen rewards [30]. Since the timing of pollen

66   release and nectar production varies across flowering plants [26,30], the time of day becomes a

67   useful contextual cue to optimise foraging behaviour [26,30].

68   Given their derived foraging behaviour it has been hypothesised that *Heliconius* have

69   evolved specific cognitive traits that support trap-lining behavior, including the ability to use the

70   time of day as a contextual cue [26,31]. However, to our knowledge, time-dependent associative

71   learning has not been reported in any Lepidoptera. In this study, we provide the first evidence

72   that *Heliconius* butterflies can form time-dependent preferences for distinct flowers, and

73   subsequently explore whether this ability is a derived trait in *Heliconius* associated with the

74   evolution of pollen-feeding.

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76

77   ## 2. Materials and Methods

78

79   Experiments were performed at the Smithsonian Tropical Research Institute in Gamboa,

80   Panama. We initially focused on *Heliconius hecale*, with secondary experiments in *Heliconius*

81   *melpomene* and *Dryas iulia*, a closely related, non-pollen feeding Heliconiini. Individuals were

82   labelled with unique IDs. Animal husbandry is described in the ESM.

83

84   *2.1. Experimental set-up*

85

86   Artificial feeders were constructed from foam sheets with an Eppendorf tube placed centrally.

87   Yellow and purple were chosen as experimental colours as they were equally approached by

88   naïve butterflies in our pilot experiments. During training, feeders contained either a 10% sugar

89   water solution with 2.5 CCF per 50 ml of critical care formula (a surrogate for pollen; rewarded

90   feeder), or a saturated quinine water solution (punished feeder). 12 artificial feeders of each

91   colour were placed on a grid of 24, with randomised positions (Figure S1). Butterflies were

92 presented with feeders for 2 hours in the morning (AM) (08:00-10:00) and 2 hours in the

93 afternoon (PM) (15:00-17:00).

94

95 *2.2. Experimental procedure*

96

97 The experiment had four phases. 1) During pre-training, freshly eclosed butterflies were fed on

98 white artificial feeders, in the AM and PM, for two days. 2) The naïve shift in colour preference

99 based on time of day was recorded prior to training, using clean, empty feeders. Due to low

100 feeding rates in the PM session, we split the initial preference test across two days. AM colour

101 preference was recorded on day 1, and butterflies were food deprived in the PM. PM colour

102 preference was performed on day 2, after food deprivation in the AM. 3) The training reward

103 structure was split such that the purple feeders were rewarded in AM and yellow feeders

104 rewarded in PM, or vice versa. This training phase lasted for 10 days. 4) During the final post-

105 training preference test butterflies were presented with clean, empty feeders for one hour in the

106 AM, followed by the reinforced AM feeders for one hour, and then clean, empty feeders for an

107 hour in the PM. To determine whether butterflies were learning the order in which they

108 encountered the reward, rather than the time of day, a proportion of butterflies had the order of

109 their AM and PM trials reversed (see supplemental material). During trials, artificial feeders

110 were filmed from above with a GoPro HERO 5 camera (Figure S1). Using this footage, we

111 scored the number of feeding attempts made by each individual.

112

113 *2.3. Training criterion*

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115 For an observed behaviour to be a consequence of learning an animal must experience the

116 reward contingency scheme [32]. Individuals were significantly less active in AM than PM

117 during training ($z = -13.11$, $n = 41$, $p < 0.01$, figure S1A). As a consequence, some individuals (n

118 $= 11$) either did not attempt to feed from both feeders in AM or, more commonly, PM during

119 training, or did not make any foraging attempts during a final test session, and were removed

120 from further analyses. We analysed the remaining data in two ways. First, we ran models

121 including all remaining individuals. Second, following previous learning studies [33–35], we

122 established a training criterion. As we are interested in whether time-dependent memories are

123   formed and can therefore guide behaviour in the absence of the reward, we identified individuals

124   that correctly adjust their behaviour in AM and PM sessions during training with reinforced

125   feeders. We then asked whether these individuals demonstrate evidence of learning by behaving

126   in the same way when presented with unreinforced feeders in the post-training preference test.

127   Our training criteria was that the majority ($>50\%$) of feeding choices made by an individual in

128   the final two training days were correct in both AM and PM.

129

130   *2.4. Statistical Analysis*

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132   Data were analysed using generalized linear mixed models (GLMMs) in R [36]. We examined

133   the influence of time on: (a) activity levels, measured as total foraging attempts, using GLMM

134   with a Poisson distribution; (b) shifts in proportional colour preference when naïve, using

135   GLMM with a binomial distribution; (c) shifts in proportional colour preference when trained,

136   using GLMM with a binomial distribution, and presentation order in the final test included as a

137   predictor. Across all models we included identity as a random effect. As individuals were trained

138   and tested in groups of 8-13 within a single cage, where possible, a random effect of cage was

139   also included to control for group level cage effects. We ensured all models fit their assumptions

140   with the R package DHARMa [37] (see ESM).

141

142   ## 3. Results

143

144   *3.1.* Heliconius *butterflies can learn time-dependent associations*

145

146   Across the *H. hecale* that experienced the full training set, there was no significant effect of the

147   time of day on naïve colour preferences ($z = 0.90$, $n = 30$, $p = 0.36$), and no overall effect of time

148   of day on trained colour preference ($z = -1.846$, $n = 30$, $p = 0.06$). However, there was

149   considerable variation in behavior during training, and only a subset of individuals ($n = 16$)

150   passed the training criterion (Figure S2). Prior to training, both butterflies that met the training

151   criterion, and those that did not, showed no significant shift in colour preference throughout the

152   day ($z = 0.33$, $n = 16$, $p = 0.73$ and $z = 1.15$, $n = 14$, $p = 0.24$ respectively).

153   Once trained, however, individuals that passed the training criteria showed a significant

154   effect of the time of day on colour preference (z = -2.24, n = 16, p = 0.02, figure 1B). On

155   average, the preference for AM rewarded colour decreased by 11% from AM to PM. The

156   presentation order of the post-training preference test (AM first vs PM first) had no effect (z =

157   0.36, p = 0.71, n =16). Among individuals that did not meet the training criterion there was no

158   shift in colour preference throughout the day after training (z = 1.05, n = 14, p = 0.29). Addition

159   of a small sample of *H. melpomene* (n = 6) support and strengthen these results (see ESM).

160

161   *3.2. Evidence time-learning is common across Heliconiini*

162

163   In a secondary experiment using *Dryas iulia*, a closely related genus within Heliconiini that does

164   not pollen feed, 12 individuals experienced the full training set, with no overall response to

165   training (z = 0.01, n = 12, p = 0.99). However, consistent with data from *H. hecale*, variation in

166   the behaviour during training resulted in only a subset of individuals (n = 6) passing the training

167   criterion. Among these individuals there was no significant effect of time of day on naïve

168   preference (z = 1.67, *n* = 6, *p* = 0.09), but post-training there was a significant effect of time on

169   colour preference, with preference for in the AM rewarded colour decreasing by 40% from AM

170   to PM (Fig 2B, z = -9.334, n = 6, p < 0.001). Individuals that did not reach the training criterion

171   show no effect of time before training (z =0.437, n= 6, p = 0.66), and show a significant shift in

172   the incorrect direction post-training (z = 7.354, n = 6, p < 0.001).

173

174   **4. Discussion**

175

176   In this experiment, we demonstrate that *Heliconius* butterflies can use time as a context for

177   making foraging decisions. The observed shift in preference here is similar in magnitude to

178   observed temporal variation in floral visits by wild *Heliconius* [30]. Time-dependent learning

179   and trap-lining can occur via an ordinal or a circadian timing mechanism [38]. Given

180   presentation order has no effect on our results, we find no support for ordinal learning as an

181   explanation, indicating the possible presence of a circadian memory. While suggestive of a

182   circadian mechanism, our data do not confirm the presence of an endogenous mechanism, as it is

183   possible butterflies are responding to external cues that covary with the time of day (*e.g.* light

184   levels, sun position). Nevertheless, the functional consequences of time learning by either

185   mechanism are similar. To the best of our knowledge, these results provide the first experimental

186   evidence of time-dependent learning in Lepidoptera.

187   The dietary innovation of pollen feeding in *Heliconius* butterflies has had major

188   implications for their life history and ecology [28,39]. For most butterflies, reproductive output is

189   constrained by the proteins collected during the larval stage [40]. Pollen feeding provides adult

190   *Heliconius* with a reliable source of protein, leading to a pronounced delay of reproductive

191   senescence [41]. Being able to efficiently exploit the sparsely distributed pollen-rich flowers is

192   therefore critical to reproductive success in *Heliconius* [41]. Competition for pollen resources

193   can be pronounced, and some *Heliconius* will forage early in the morning and actively defend

194   flowers against other butterflies [23], suggesting selection may have favoured cognitive

195   mechanisms that increase foraging efficiency. On this basis, it has been suggested that

196   *Heliconius* may have acquired the ability to use time as a foraging cue in the context of pollen-

197   feeding [26]. Indeed, our experiments confirm *Heliconius* can use time as a foraging cue.

198   However, we also show that a non-pollen feeding relative, *Dryas iulia*, has the same capacity.

199   This suggests that an ability to learn time-dependent associations did not evolve in response to

200   selection for trapline foraging, and pre-dates the origin of pollen-feeding. Although our sample

201   size for *Dryas* is smaller than for *Heliconius*, the proportion of individuals passing the training

202   criterion and the pattern of results are highly consistent. While data on the foraging behavior of

203   *Dryas* in the wild is limited, they have no known foraging specialisations that are not seen in

204   other groups, and have lifespans typical for tropical species [42]. It is therefore reasonable to

205   suggest that the ability to use time as a contextual foraging cue may be widespread across

206   butterflies.

207   Previous work shows that time learning is prevalent among social Hymenoptera, where

208   allocentric foraging provides an ecological context for using time cues in the context of a

209   consistent foraging landscape [43–45]. *Heliconius* have converged on several foraging

210   behaviours observed in these species, and also share dramatically expanded mushroom bodies, a

211   region of the insect brain responsible for learning and memory [39]. Time-dependent memory

212   acquisition is also reported in cockroaches [46], a third clade associated with mushroom body

213   expansion [47]. This could be seen as indicating that the ecological challenges associated with

214   learning foraging sites exert selective pressures favouring neuroanatomical elaboration

215 supporting specialised cognitive abilities, like time learning [21,47–50]. However, our data on
216 *Dryas* suggest that elaborated mushroom bodies are not necessary for the time learning abilities
217 displayed in these taxa. This is further supported by the fact that *Drosophila*, which have
218 substantially smaller mushroom bodies, can also learn time-dependent olfactory associations
219 [14]. Therefore, the neural basis of integrating time information with foraging cues may be
220 relatively simple. Integrating time and place memories may be more complex than forming these
221 associations in isolation, as hypothesised in hummingbirds [48,51]. However, time learning is
222 likely to be an important precursor for temporally and spatially faithful foraging. Hence, the pre-
223 existence of this trait may have helped facilitate the evolution of trap-lining, and the transition to
224 pollen-feeding in *Heliconius*.

225   The ability to form time-dependent associations may have wider ecological effects. If a
226 pollinator has a time learning ability, sympatric plant species can coexist by sharing, rather than
227 competing for, the same pollinator by temporally partitioning pollen or nectar rewards [28]. This
228 effect may explain observed divergence in the nectar/pollen release schedules of *Psiguria*
229 flowers, a preferred pollen resource for *Heliconius* [26]. A similar phenomenon is observed in
230 bees and *Dalechampia* flowers, adding support to the idea that time learning abilities have
231 implications for ecological diversity [28].

232   Overall, our results support the importance of temporal predictability in resources, rather
233 than allocentric foraging or pollen-feeding, in promoting an ability for time learning. This is
234 supported by the presence of time learning in both *Dyras* and *Heliconius*, and both pollinivorous
235 and nectarivorous Hymenoptera. Moreover, time learning does not seem to be associated with an
236 expansion of insect memory centres. Whether butterflies use internal or external cues to register
237 time of day remains an open question for future study.

238

Ethics

240 This work was carried out under permission from the Ministerio del Ambiente, Panama (permit
241 number: SE/AP-14-18).

242

Data accessibility

244 Data and R scripts are available from the Dryad Digital Repository:
245 (https://doi.org/10.5061/dryad.c59zw3r4m).

246

Author's contributions

248 MWT, FJY, SHM conceived and designed the experiment. MWT collected and analysed the

249 data, and wrote the paper, with practical support and input from FJY and WOM, under the

250 supervision of SHM.

251

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Competing interests

254 We declare no conflict of interest.

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407 **Figure legends**

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409 Figure 1: Data from the colour preference trials of *H. hecale* individuals who met the training

410 criterion. Artificial feeders were new, empty, and unreinforced in both cases. (A) Naïve

411 preferences of butterflies in the morning and afternoon. (B) Colour preferences of butterflies

412 from (A) after training. Grey lines connect individuals across time periods. Data are presented as

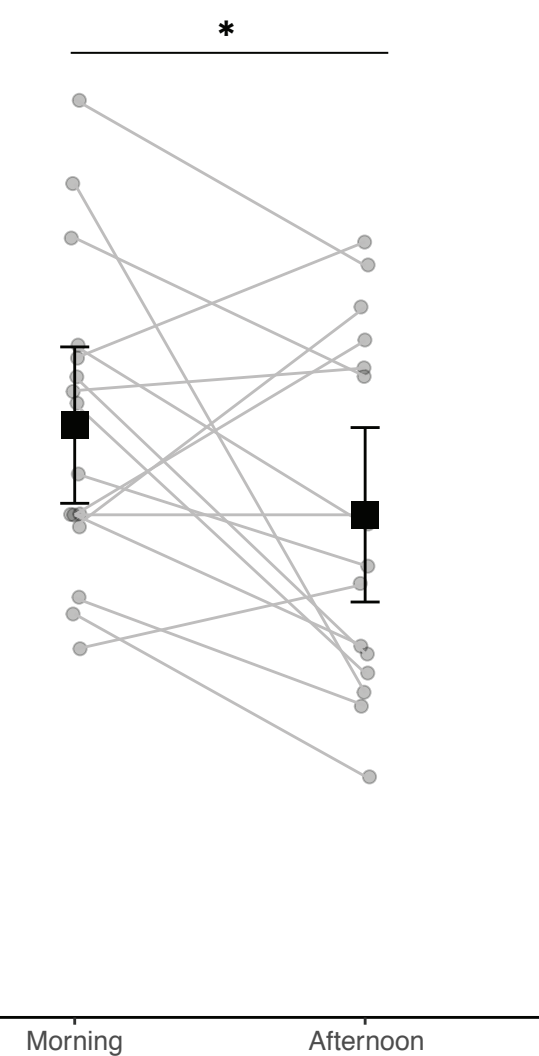413 means ± SE. * indicates P <0.05

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415 Figure 2: Data from the colour preference trials of *D. iulia* individuals who met the training

416 criterion. Artificial feeders were new, empty, and unreinforced in both cases. (A) Naïve

417 preferences of butterflies in the morning and afternoon. (B) Colour preferences of the butterflies

418 from (A) after training. Grey lines connect individuals across time periods. Data are presented as
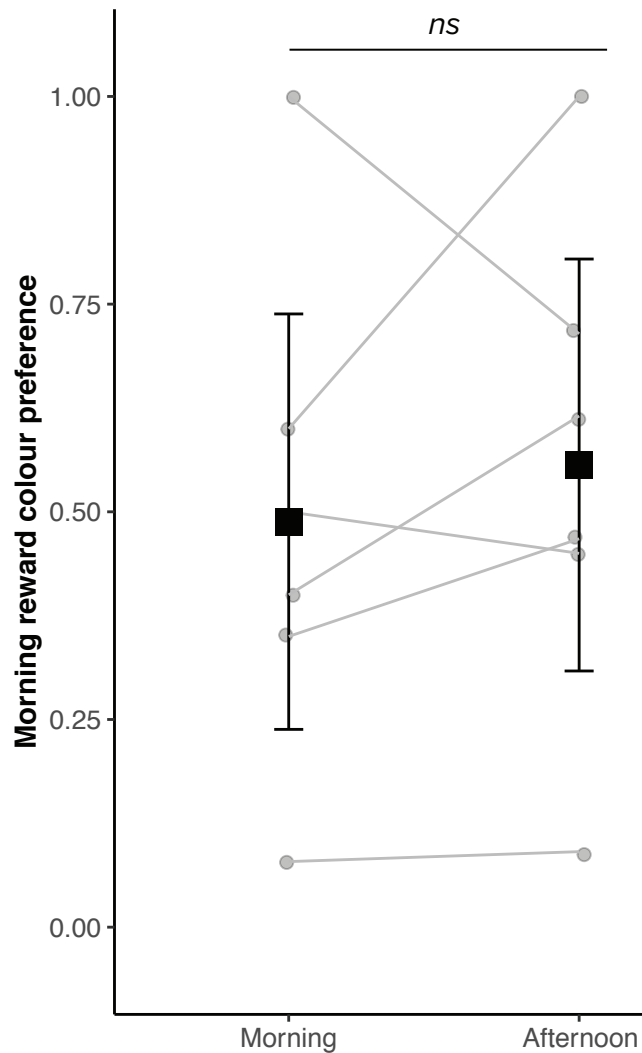
419 means ± SE. ** indicates P<0.01

(A) Naive shift in preference
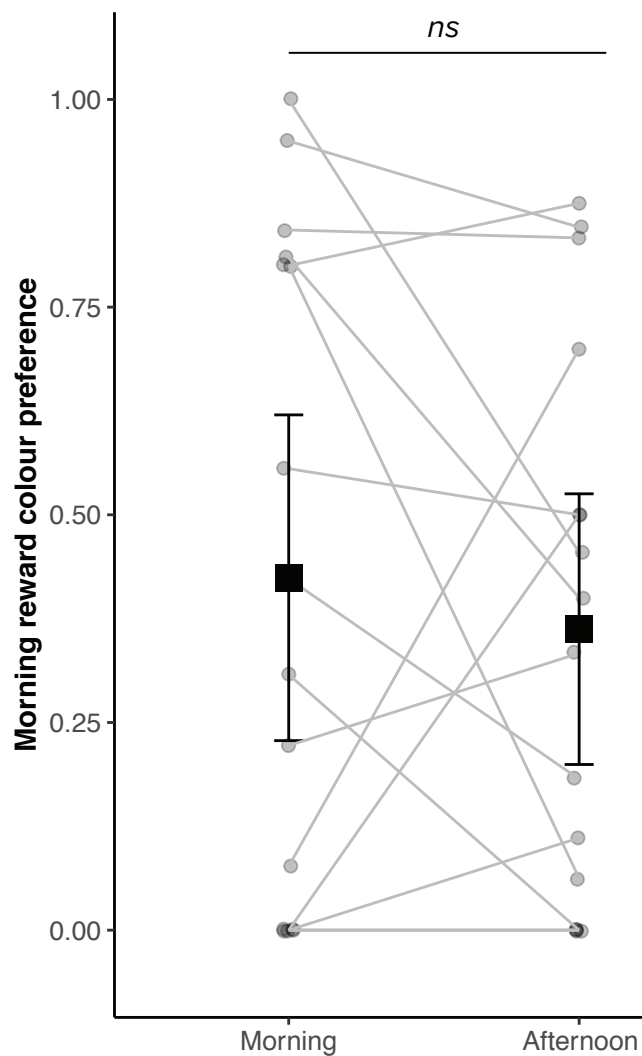
(B) Trained shift in preference

(A) Naive shift in preference

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(B) Trained shift in preference

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Morning reward colour preference

Time of Day