1 **Title: Relationship Between Inter-individual Variation in Circadian Rhythm and Sociality: A case Study Using**

2 **Halictid Bees**

3 **Author information: Sofía Meléndez Cartagena [1], Carlos A. Ortiz-Alvarado[1], Patricia Ordoñez [2],Claudia S.**

4 **Cordero-Martínez[1], Alexandria F. Ambrose[3] , Luis A Roman Lizasoain[1], Milexis A Santos Vega[1], Andrea V**

5 **Velez Velez[1], Jenny P. Acevedo-Gonzalez[1], Jason Gibbs[4], Theodora Petanidou[5], Thomas Tscheulin[5], John T.**

6 **Barthell[6], Victor H. González[7], Tugrul Giray[1], José L. Agosto-Rivera[1]**

7 1. Department of Biology, University of Puerto Rico Rio Piedras, San Juan, PR

8 2. Department of Computer Science, University of Puerto Rico Rio Piedras, San Juan, PR

9 3. Department of marine and coastal biology,Rutgers University, New Brunswick, NJ

10 4. Department of Entomology, University of Manitoba, Manitoba, CAN

11 5. Department of Geography, University of the Aegean, Mytilene, GR

12 6. Department of Biology, University of Central Oklahoma, Oklahoma, USA

13
14 7. Undergraduate Biology Program and Department of Ecology and Evolutionary Biology, University of Kansas, Kansas, USA

15 **Correspondence:**

16 Corresponding author: Sofía Meléndez Cartagena

17 Email: sofia.melendez.cartagena@gmail.com

18 **Abstract:**

19 The bee family Halictidae is considered to be an optimal model for the study of social evolution due to its

20 remarkable range of social behaviors. Past studies in circadian rhythms suggest that social species may express more

21 diversity in circadian behaviors than solitary species. However, these previous studies did not make appropriate

22 taxonomic comparisons. To further explore the link between circadian rhythms and sociality, we examine four

23 halictid species with different degrees of sociality, three social species of *Lasioglossum*, one from Greece and two

24 from Puerto Rico, and a solitary species of *Systropha* from Greece. Based on our previous observations, we

25 hypothesized that species with greater degree of sociality will show greater inter-individual variation in circadian

26 rhythms than solitary species. We observed distinct differences in their circadian behavior that parallel differences

27 across sociality, where the most social species expressed the highest inter-individual variation. We predict that

28 circadian rhythm differences will be informative of sociality across organisms.

29   **Keywords:** Circadian Rhythms, Halictidae, Sociality, Behavior

30   **Declarations:**

40   **Conflicts of interest/Competing interests:** The authors declare no conflicts of interest.

41   **Availability of data and material**: All data analyzed during this study is included in its supplementary information

42   files.

43   **Authors' contributions:**

44   All authors contributed to the study conception and design. Material preparation, data collection and analysis were

45   performed by Sofía Meléndez Cartagena, José L. Agosto-Rivera, Carlos A. Ortiz-Alvarado, Claudia S. Cordero-

46   Martínez, Alexandria F. Ambrose , Luis A Roman Lizasoain, Milexis A Santos Vega, Andrea V Velez Velez. The

47   first draft of the manuscript was written by Sofía Meléndez Cartagena and all authors commented on previous

48   versions of the manuscript. All authors read and approved the final manuscript.

49   **Significance Statement:**

50   Circadian rhythms differ across solitary and social organisms. One important feature we discovered is that

51   in highly eusocial insects there was a high level of individual variation in circadian activity. We took advantage of

52   varying levels of sociality within halictids and examined the circadian rhythms of multiple members of the genus

53   *Lasioglossum* and of a *Systropha* species. This work is the first report to link interindividual variation in circadian

54   rhythms with the degree of sociality: interindividual variation increases with increased level of sociality. This study

55   invites investigating the potential role of circadian rhythm variation in social organization.

Introduction:

57   Understanding the evolutionary link between solitary and eusocial lineages, and their adaptive behaviors,

58   such as those expressed in reproduction and brood care, is a perennial question in insect evolutionary biology (Toth

59   and Rehan 2017). Insects, and in particular hymenopterans, have been useful in observing how sociality is related to

60   other types of behaviors, such as competitor effects (Peters et al. 2017). A potential behavior to be evaluated in

61   relation to sociality is circadian rhythm, as it has been proposed to be governed by demands arising from sociality not

62   only in insects but also in mammals (Mistlberger 2004; Giannoni-Guzman et al. 2014; Beer and Helfrich-Förster

63   2020).

64   Circadian rhythms can be viewed as a biological clock that most living organisms possess. These biological

65   clocks regulate processes such as gene expression, behavior, body temperature, and sleep-wake patterns. Biological

66   clocks follow a rhythm that is approximately synchronized to Earth's 24-hour rotation using signals from the

67   environment called "zeitgebers" or time givers. This process of synchronization needs active reestablishment, and it

68   is called entrainment (Roenneberg et al. 2003).

69   Circadian rhythms have been studied in a wide range of organisms, from plants, invertebrates, birds to

70   mammals (Helm and Visser 2010). The traditional model animal to study this phenomenon is the fruit fly, *Drosophila*

71   *melanogaster*, where biological clocks are described at the molecular level (Dubowy and Sehgal 2017). Although this

72   model has been pivotal to the understanding of circadian rhythms, the lack of genetic diversity in the fruit fly reduces

73   the relevance of the model because it limits questions regarding individual differences in rhythms.

74   Past studies, such as those of Bloch et al. (2001) and Moore et al. (1998), revealed that the rhythmicity of

75   honey bees changed with age. Additionally, Giannoni-Guzman et al. (2020) showed that foragers in the wild display

76   discrete categories that suggest temporal shift work. An earlier study from Giannoni-Guzman and colleagues (2014)

77   compared the endogenous period of three different variants of honey bees (*Apis mellifera carnica*, *Apis mellifera*

78   *caucasica* and *Apis mellifera gAHB*) as well as similarly sized insects from different orders and families. They found

79   that honey bees and paper wasps (*Polistes crinitus* and *Mischocyttarus phthisicus*) had a larger degree of circadian

80   period variation within the population in comparison to *D. melanogaster*. The authors mentioned several possible

81   explanations for their observations, one of them being demands of sociality. In a more recent study by Beer and

82   Helfrich-Förster (2020), they explore this connection further and note that the development of the circadian circuitry

83   varies between a eusocial (*Apis mellifera*) and a solitary species (*Osmia bicornis)*. In particular, they observe that

84   eusocial individuals do not display circadian locomotion at adult emergence, while the solitary individuals emerge

85   displaying a fully rhythmic circadian rhythm; These differences are attributed to their opposite levels of sociality.

86   However, because these two past studies were done with species spanning from different taxonomic groups, it would

87   be difficult to support their claims without taking phylogeny into account. Nevertheless, these works do give a basis

88   to ask how circadian rhythms vary and are an integral part of the survival strategy and organization of these animals.

89   Moreover, it makes us consider that the level of sociality in different organisms may play a role in their daily activity

90   patterns. An important emerging feature is that in social insects, be it groups of individuals as defined by their age and

91   job (Moore et al. 1998, Bloch et al. 2001), or individuals in the same age and task group (Giannoni-Guzman et al.

92   2014, 2020), exhibit differences in their circadian rhythms with functional significance for their sociality. We

93   hypothesize that with increasing levels of social organization, as can be measured in size of the social group, greater

94   levels of individual variation in circadian rhythms will be observed.

95   We tested this hypothesis of socially increased individual variation, by examining circadian rhythms of

96   foragers from differently social halictid bees. Halictidae (Hymenoptera) is a bee family considered to be a great model

97   for the study of social evolution due to its exceptional diversity in respect to social behavior within and among species

98   and populations (Schwarz et al. 2007). *Lasioglossum* Curtis is one of the two genera in the tribe Halictini that displays

99   eusocial behavior, but also includes solitary representatives and a range of intermediate social categories (Danforth et

100   al. 2003; Gibbs et al. 2012). Additionally, past studies have shown plasticity in the social behavior even among

101   populations of the same species (Eickwort et al. 1996; Field 1996; Field et al. 2010; Richards et al. 2003; Soucy and

102   Danforth 2002; Richards 2000). Depending on environmental conditions, such as elevation, latitude and seasonality,

103   halictid bees might display different modes of sociality. Species with social nests may revert to solitary behavior at

104   high latitudes and altitudes (Eickwort et al. 1996; Packer et al. 1983; Field et al. 2010) or based on access to mates

105   (Yanega 1988/1989). Jeanson et al. (2008) studied members of a solitary species, *Lasioglossum* (*Ctenonomia*) sp.

106   NDA-1 and observed the results of having them nest in pairs. They finally observed that after some time together, the

107   individuals in the nest started to show signs of division of labor. This plasticity and diversity of behavior in addition

108   to the close taxonomic relation, makes Halictidae an optimal model for observing the relationship between sociality

109   and circadian rhythm (Bloch and Grozinger 2011).

110   To better understand association of sociality with individual variation in circadian rhythms, we have set out

111   to document the rhythm in four species of halictid bees that span a gradient of social complexity. *Systropha curvicornis*

112   (Scopoli) (Halictidae: Rophitinae), a solitary pollinator specialist (Grozdanić and Mučalica 1966) considered

113   ancestrally solitary within the family Halictidae (Patiny and Michez 2007; Patiny et al. 2008; Danforth et al. 2008),

114   and three species of *Lasioglossum* (*L. ferreri*, *L. enatum* and *L. malachurum*), which were selected because of their

115   varying levels of social behavior (Eickwort 1988; Wyman and Richards 2003; Gibbs 2018). Species of *Lasioglossum*

116   likely had a common ancestor capable of eusocial nesting but have reverted multiple times to other levels of sociality

117   (Danforth et al. 2003; Brady et al. 2006; Gibbs et al. 2012). *Lasioglossum* (*Dialictus*) *ferrerii* (Baker) and *L.* (*Dialictus*)

118   *enatum* (Gibbs) occurs in the Caribbean whereas *L. malachurum* across Europe; the first species nests communally,

119   that is each individual contributes to nest construction and reproduction (Michener 1974; Eickwort 1988). Although

120   *L. enatum* has not been thoroughly studied, this species is part of a species complex that includes weakly eusocial

121   species. Namely, *L. gemmatum* (Smith) and *L. parvum* (Cresson) from Jamaica and the Bahamas, which exhibit

122   reproductive division of labour (Eickwort 1988). There is no evidence of morphologically defined castes beyond

123   reproductive status in these two species and thus we assumed this is likely the case for *L. enatum* in Puerto Rico,

124   where we conducted our experiments. In contrast, *L. malachurum* (Kirby) is an obligately eusocial species with

125   morphologically well-defined queen and worker castes (Richards 2000; Wyman and Richards 2003). *Lasioglossum*

126   *malachurum* is known to display varying degrees of behaviors depending on location (Richards 2000). In Lesbos,

127   Greece, where we studied this species, they were observed to exhibit a facultatively eusocial behavior (Wyman and

128   Richards 2003). In summary, the level of sociality in the four species goes from lowest to highest: solitary *Systropha*

129   *curvicornis*, communal *Lasioglossum ferreri*, weakly eusocial *Lasioglossum enatum*, and the eusocial *Lasioglossum*

130   *malachurum*. The socially increased individual variation hypothesis predicts individual variation of foragers from

131   each species to increase from solitary to eusocial. Alternatively, individual variation could be the result of ecological

132   differences such as differences in robustness of time givers in different environments. Two of the halictids examined

133   here are from a tropical zone (a communal and an eusocial) and two are from a temperate zone (a solitary and an

134   eusocial).

135   The social plasticity of *Lasioglossum* and its potential as a model for social evolution leads us to believe that

136   observing this group of bees can give invaluable insight on how social behavior affects biological clocks. To test if

5

137   sociality increases individual variation, we captured the forager bees as they were visiting flowers and observed their

138   circadian variability in the laboratory using constant conditions.

139   **Methods and Materials:**

140   *Study sites*

141   *Puerto Rico:*

142   *Lasioglossum ferrerii* and *L. enatum* were captured using 15 mL falcon tubes from flowers at the Balneario

143   de Luquillo parking lot (18.38706 N 65.72517W, 3 Meters) in Puerto Rico. This site is characterized by having many

144   vine-type plants, high vegetation density, and it is located right next to a road with *Momordica charantia* being the

145   most abundant (Figure 1.A and 1.B). Most bees were caught between 8:00 and 12:00 h at the flowers of *Momordica*

146   *charantia* L. (Cucurbitaceae), *Sida acuta* Burm.fil. (Malvaceae)*,* and *Bidens alba* (L.) DC. (Asteraceae). We also

147   observed them visiting *Euphorbia heterophylla* which has not been reported in previous literature. Collections took

148   place during the months of December, January, March, and August. In total we collected 36 bees, 26 of which were

149   *L. ferrerii* and 10 were *L. enatum*.

150   *Greece:*

151   *Systropha curvicornis* and *L. malachurum* bees were collected between 6:00 and 9:00 h from flowers of

152   *Convolvulus arvensis* (Convolvulaceae) that were growing on a recently cut wheat field in Skala Kallonis (39º 10'N

153   26º 20'E, 0 Meters) on the Island of Lesbos, Greece. We used 15 mL falcon tubes to catch bees in the field, which

154   would house the individual for the duration of the experiment. Sampling was conducted on July 3 of 2017. From this

155   sampling 118 bees were *L. malachurum* and 34 were *S. curvicornis.*

156   *Laboratory settings*

157   After collection in the falcon tubes, bees were provided with food that lasted for the whole of the observation

158   period. The food recipe we used varied between the studies in Puerto Rico and Greece (As explained below). The

159   main nutrient for both recipes was sugar and therefore are nutritionally comparable. However the agarose based recipe

160   (Puerto Rico) was more convenient in terms of ease and speed of preparation due to the fact that an independant water

161   system was not necessary.

162   Food preparation varied by locations as follows: In Puerto Rico, for every 0.89 ml of water, 1 g of sucrose

163   and 0.1 g of agarose were used. The water was heated in a stirring plate with a magnetic stirrer placed at the bottom.

164   We added the sucrose first to the solution, and when it was dissolved, the agarose was incorporated. The solution was

165   left stirring until it turned into a lighter color while mindful of not letting the solution heat too much, or part of the

166   volume would be lost. As a form of assurance, we made 3 ml more than what was expected to be used. After all solids

167   were diluted, we quickly pipetted 1 ml into the bottom of a 15 ml centrifuge tube, being mindful of not letting it splash.

168   Once all of the tubes had their portion of the solution, they were allowed to reach room temperature and finally they

169   were refrigerated. The final product was a gel that could be kept refrigerated until the day it needed to be used as long

170   as it did not dehydrate.

171   In Greece, captured *S. curvicornis* and *L. malachurum* were fed with ApiYem (Namik Kemal University with

172   Kosgeb R&D Innovation Project) which is a commercial bee feed composed of 78.5% sugar and 21.5% invert syrup.

173   Food was placed in the cap-end of each tube and a damp cotton was placed in the other end of the tube to provide

174   water to the bees. The water supply was refilled every 2–3 days. Resources were provided ad libitum during the

175   complete running period of the experiment.

176   *Locomotor activity monitoring*

177   Each bee was monitored individually for at least seven days in the falcon tube in which they were captured.

178   The tubes were plugged using cotton balls to let air circulate. These tubes were then placed into TriKinetics'

179   Locomotion Activity monitors (LAM16) that, in turn, were put inside incubators that were set to constant conditions.

180   In Greece the temperature was 26 , humidity 78%, constant darkness. In Puerto Rico the temperature was 30, humidity

181   65%, constant darkness. The differences in the environmental chamber conditions were set to resemble the average

182   daytime parameters at each location.

183   *Species Identification*

184   The individuals caught in Puerto Rico were identified using Gibbs (2018). Samples collected in Greece were

185   identified by an in-field expert, Victor H. Gonzalez (University of Kansas).

186   **Data processing and analysis**

187   ***Circadian Analysis***

188   Circadian rhythm and locomotor activity for our subjects were analyzed using the MATLAB toolboxes

189   developed in Jeffrey Hall's laboratory (Levine et al. 2002). The outputs provided data on the individual's locomotor

190   activity throughout the experiment in the form of an actogram, average activity plot, and an autocorrelation that also

191   calculates rhythm strength.

192   To test if the observed differences in circadian patterns across species were statistically significant, we

193   applied a Brown-Forsythe one way ANOVA with a Dunnett's T3 multiple comparisons test using GraphPad Prism

194   version 8.4.3 for Windows, GraphPad Software, San Diego, California, USA, www.graphpad.com. The variables

195   taken into consideration for this was time, species, individuals, and interspecies variation.

196   **Results:**

197   During the Summer of 2017 on the 3rd of July between the hours of 6:00 am and 9:00 am, *S. curvicornis*

198   and *L. malachurum* were caught as they visited the flowers of Convolvulus *arvensis o*n the island of Lesbos. They

199   were transported from the field to the laboratory and placed inside an incubator for 10 days of which 8 were in

200   constant conditions (26 °C and 57 % humidity) with the purpose of characterizing their intrinsic biological

201   clockDuring this collection 118 bees were *L. malachurum* although only 98 survived until the end and 34 were *S.*

202   *curvicornis* of which only 4 females survived the study period.

203   Figure 2.A illustrates the average of individuals evaluated under constant conditions of the Greek, solitary

204   and specialist pollinator *S. curvicornis*. Its period runs slightly short at approximately 22 hours, with the peak of its

205   activity in the early morning and an average rhythm strength of 4.4. The population average is consistent with the

206   individuals examined, as illustrated in Figure 2.B; a randomly selected individual looks fairly similar to the activity

207   plotted for the population average. The average for period was 22.75 with a standard deviation of 0.41, Rhythm

208   Strength had an average of 4 and standard deviation of 0.707.

209   The consistency displayed by our population of female *S. curvicornis* is contrasted with the diversity

210   observed in the other 3 species analyzed in this study. This is especially true for the eusocial *L. malachurum*, for

211   whom after careful evaluation of the data we had to create a classification schematic (Figure 3) to appropriately

212   describe the phenotypes being displayed by the population. The population average shows that *L. malachurum,* as a

213   species, has a perfectly circadian 24-hour period under constant dark conditions. Peak average activity of *L.*

214   *malachurum* is at 6:00 h, with no clear rest periods when all individuals are averaged. When examined individually,

215   we found five distinct patterns of circadian activity patterns (Figure 3.A and Figure 4). These patterns can be divided

216   into 2 large branches (Figure 3.A), those that are rhythmic and those that are arhythmic i.e, individuals with uniform

217   distribution of activity. Rhythmic individuals varied in the amplitude of their activity rhythm and therefore were

218   classified as strong or weakly rhythmic. Moreover, both strong and weak categories are subdivided into unimodal or

219   bimodal based on the number of activity peaks per day. For example, a bimodal individual is active during two

220   different instances of the day like the morning and the afternoon (Figures 4.B and 4.C), while a unimodal individual

221   is mostly active during a set time of the day (Figures 4.D and 4.E).

222   Strongly rhythmic individuals (either unimodal or bimodal) constituted 41% of individuals. These patterns

223   are recognized by a strong Rhythm Strength (RS)(Figures 4.B.iii and 4.D.iii) on average higher than 2.67 and clear

224   rest and active periods both in the double plotted actogram (Figures 4.B.i and 4.D.i) and the average activity

225   plot(Figures 4.B.ii and 4.D.ii). Weak rhythmicity (Figure 4.C and 4.E) was observed in 21.6% of individuals and

226   they were characterized by having RS values (Figure 4.C.iii and 4.E.iii) that average on 1.79, but their actograms

227   (Figure 4.C.i and 4.E.i) do not show a clear pattern of locomotor activity. Finally, 38% of individuals were

228   arrhythmic. Both the double plotted actogram (Figure 4.F.i) and the average activity plot (Figure 4.F.ii)for

229   arrhythmic bees do not have any discernible daily pattern of activity or inactivity. Often the autocorrelation (Figure

230   4.F.iii) does not return any values.

231   On February 19, 2020, between 8:00 am and 10:00 am, at the Balneario de Luquillo (Figure 1.A and B), 36

232   bees were captured as they visited *Bidens alba*, *Momordica charantia* and *Sida acuta*. Bees were captured and

233   monitored individually in one tube each (modified from Giannoni-Guzman et al. 2014). In the laboratory, the bees

234   were placed inside an incubator for seven days in constant conditions so we could characterize their intrinsic

235   biological clock.

236   Of the 36 bees captured, 26 were identified as *Lasioglossum ferrerii* (Figure 1.C and 1.D) and ten as

237   *Lasioglossum enatum* (Figure 1.E and 1.F). Only 22 *L. ferrerii* and 8 *L. enatum*, survived the entire observation

238   period and were used for analysis. The average peak of circadian activity for *L. ferrerii* is between 6:00–7:00

239   (Figure 5.A.ii) with a 23 hour period (Figure 5.A. iii), making it short. Individuals fell into two categories, 50% were

240   strongly rhythmic and 50% were weakly rhythmic (Figures. 5B and 5C). The peak of average activity for *L. enatum*

241   is from the fifth to the seventh hours of the day, with a circadian period of 23.8 hours (Figure 6.Aiii), just slightly

242   short of a day. The average peak of activity for *L. enatum* was 6:00-7:00 (Figure 6.A.ii). *L. enatum* also had three

243   patterns of activity with similar characteristics to that of the umbrella categories for *L. malachurum*, and we saw it

244   fit to categorize them in a similar fashion (Figure 6). 12.5% of the observed population fell into the strongly

245   rhythmic category, 25% in the weak rhythmic category and 62.5% in the arrhythmic category.

246   In summary, all four of the described species followed unique patterns of behavior (Figure 7.A)

247 characterized by the amount of interindividual variation. *Systropha curvicornis* was the species with the least

248 amount of observed interindividual variation in its daily activity patterns, followed by *L. ferrerii* with two distinct

249 patterns of behavior, then *L. enatum* with 3 and lastly *L. malachurum* with 5.

250 *Cross species comparison of observed circadian parameters*

251   Average activity was only significant between *L. ferrerii* and *L.malachurum* with a p-value of 0.0016

252 (Figure 7.B). Circadian Period (Figure 7.C) on the other hand showed differences between *S. curvicornis* and *L.*

253 *ferrerii* with a p-value of 0.0102 as well as *L. malachurum* and *L. ferrerii* with a p-value of <0.0001. Lastly, with

254 even more differences still, Rhythm Strength (Figure 7.D) presented differences between S. *curvicornis* and *L.*

255 *enatum* (p-value = 0.0014), *S. curvicornis* and *L. malachurum* (p-value = 0.0177), *L. ferrerii* and *L. enatum* (p-value

256 = 0.0056) and finally, *L. ferrerii* and *L. malachurum* (p-value = 0.0259).

257 **Discussion:**

258   This study lays the groundwork for the use of halictid bees for cross species comparisons of circadian

259 rhythms. Consistent with our hypothesis, we found that greater degrees of sociality are associated with larger

260 individual differences of circadian rhythms within a population.

261   The solitary specialist, *S. curvicornis* as observed in this work, suggests that at least for the females, the

262 population is consistent, displaying a single circadian activity phenotype (Figure 7.A). Activity of these bees is

263 highly rhythmic and shows little variation across samples with an average RS of 4.4 and the peak of activity appears

264 to be near the hour 6 of the day. Overall, the species exhibits a short period phenotype under constant darkness. This

265 high degree of rhythmicity might be due to *S. curvicornis'* evolutionary history as a foraging specialist of *C.*

266 *arvensis*, which blooms for a brief period during the morning, a pattern described for another closely related species

267 (*S. planidens*: Gonzalez et al. 2014). A rigorous internal clock is important to be able to anticipate the time when

268 resources are available. For example, the immediate development of *Osmia bicornis'* circadian rhythm (Beer and

269 Helfrich-Förster 2020) may be related to nurishment accessibility, as it has been shown in the past that large

270 quantities of pollen are the key to proper larvae development rather than diversity of pollen (Radmacher and Strohm

271 2010). All three of these species (*S. curvicornis*, *S. planidens* and *O. bicornis*) lead solitary lifestyles and

10

272   consequently must assure the survival of their progeny in an individual manner. A strong circadian rhythm can

273   ensure that a female may find sufficient resources efficiently to feed its young.

274   All three species of *Lasioglossum* examined were shown to have more than one distinct pattern of circadian

275   activity. The most diverse of the three was the facultatively eusocial *L. malachurum* (Figure 7.A) with 5 distinct

276   circadian behaviors. Two of the sub-categories of these behaviors fall under the strongly rhythmic category, which

277   we are calling binomial and unimodal. These rhythmic categories are characterized for having an easy to distinguish

278   pattern in the actogram, clear rest/activity periods in the average activity plot, and an RS higher than one. Another

279   two of the sub-categories fall under the weakly rhythmic umbrella. This umbrella, just like the strongly rhythmic

280   category, can be divided into bimodal and unimodal. These categories can be identified by an actogram with no

281   clear pattern, an average activity plot with more or less clear rest/activity pattern, and an RS larger than one. Lastly,

282   there is the arrhythmic category where no discernible pattern can be pinpointed in the actogram nor in the average

283   activity plot and its RS is less than one. A conceptual map on how these categories are identified can be found in

284   Figure 3.A.

285   To make descriptions comparable across species, we used the same metrics to categorize the other two bees

286   examined in this study. In categorizing *L. ferrerii* and *L. enatum*, our categories worked as a good basis. *L. ferrerii*

287   only had two distinguishable patterns: rhythmic and noisy rhythmic (Figure 7.A). We decided to change the name

288   from weakly rhythmic to noisy rhythmic because it is a better descriptor (Figure 3.B). Similarly, *L. enatum*, which

289   lives in the same environment as *L. ferrerii*, has 3 distinguishable categories (Figure 7.A). These categories are

290   rhythmic, noisy, rhythmic and arrhythmic. Contrary to *L. ferrerii*, *L. enatum* expressed 5 individuals in the

291   arrhythmic category. Taking into consideration that both of these species of bees were caught in the same

292   environment, and they belong to the same genus, the results suggest that something other than environmental

293   variables are behind these differences.

294   The difference in expression of circadian patterns between *L. ferrerii* and *L. enatum* could be explained by

295   competition. Both of these species share the same niche in Luquillo, to the point of them being caught in the same

296   flowers during the same range of time. Having a slight difference in rhythmicity can lower the possibility of

297   temporal competition when foraging. *L. ferrerii* on average would be active from 5:00 am to 10:00 am, while

298   average time of activity for *L. enatum* would be from 3:00 am to 8:00 am. Due to that two-hour disphase, it would

11

299   appear to be less likely that bees from these two species try to visit a flower simultaneously, yet their schedules still

300   have some overlap. These observations are echoed by another study conducted in Greece where they demonstrated

301   that 3 species of carpenter bees (*Xylocopa* spp.) that share the same resources have different circadian rhythms when

302   measured under natural field conditions and also in artificial constant and oscillating conditions (Ortiz-Alvarado et

303   al. *in rev.*).While the solitary *Xylocopa* species have interspecies variation in their circadian rhythms, two out of the

304   3 examined in Ortiz-Alvarado et al. follow a similar pattern as S. curvicornis, were there isn't much if any individual

305   differences in the populations examined. Therefore in that particular case, competitor effects can explain the

306   differences in rhythm across species, but in the case of *L. ferrerii* and *L. enatum* it cannot explain the individual

307   differences observed at the species level.

308   At a higher level looking at the statistical analysis of all 4 halictid bees (Figure 7), some interesting patterns

309   can be noted. In terms of average activity (Figure 7.B), there was only a difference between *L. ferrerii* and *L.*

310   *malachurum*, none of the other possible combinations of differences occurred. However, the length of the whiskers

311   in the box plots for both *L. enatum* and *L. malachurum* does suggest a level of diversity at the intraspecies level and

312   could be reflective of the number of circadian behaviors observed in these species.

313   When analyzing the circadian period, the observed differences were between *L. ferrerii* and *L. malachurum*

314   as well as *S. curvicornis* and *L. malachurum* (Figure 7.C). The latter of these pairs have shared environmental

315   conditions when the former pair does not. It is also interesting to note that *L. ferrerii* and *S. curvicornis* cannot be

316   found in the same locations, and yet they do not appear to have significantly different circadian periods, in fact for

317   the populations examined, they appear to be comparable.

318   The connection between individual differences in circadian rhythm and sociality becomes clearer still when

319   observing rhythm strength (Figure 7.D). Where those species with a lesser number of circadian phenotypes are more

320   similar to each other and likewise the ones with the most diversity are more similar to each other. In other words, *S.*

321   *curvicornis* and *L. ferrerii* were both significantly different to *L. enatum* and *L. malachurum,* but not to each other.

322   Likewise, there was no significant difference between *L. enatum* and *L. malachurum*. Because there is this

323   consistency of differences that is not associated with differences in environments, we believe that the key to

324   explaining the difference in diversity of behaviors may not lay in competition, but in something more endogenous of

325   the species. Nevertheless, more data is needed.

12

326   *Lasioglossum* as a genus is well-known for having a large diversity in social behaviors. This diversity in

327 sociality may also be reflected in other types of behaviors and could be the key to explaining the individual

328 differences in circadian rhythm we observed across foragers within the species. One caveat for the present work is

329 the sample size and populations evaluated (one population for each species and low number of individuals,

330 particularly for *L. enatum*). One future direction is to identify additional populations of the same 4 species to

331 examine consistency of the presented findings. In future studies we will focus on streamlining the process of

332 describing the diverse circadian behaviors observed in a species to facilitate studies with a higher volume of

333 observations. Additionally, we will continue describing the circadian rhythm of additional species of *Lasioglossum*

334 that present social behaviors not evaluated in this study. Understanding the functional relationship between sociality

335 and rhythm will add to further understanding of mechanisms underlying social organization.

336 **Figure Legends:**

337 **Figure 1: Habitat (A-B) and Species Observed (C-F). A)** Puerto Rico study site in which *L. ferrerii* and *L.*

338 *enatum* were captured. **B)** Some of the vegetation the bees were observed visiting, with flowers belonging to the

339 families: Commelinaceae, Cucurbitaceae and Euphorbiaceae. **C)** Female of *L. ferrerii* distinguished from the male

340 by its short antenna and pointed abdomen. **D)** Male of *L. ferrerii*, distinguished by its long antennae and flat

341 abdomen. This species is known for its long head shape and metallic metasoma (Gibbs 2018). **E)** Female of *L.*

342 *enatum* distinguished from the male by its short antenna and pointed abdomen. **F)** Male of *L. enatum*, distinguished

343 by its long antennae and flat abdomen. This species is distinguished by: "tegula punctate, extended posteriorly to

344 form a small angle, mesepisternum punctate and metasoma brown" (Gibbs 2018).

345 **Figure 2**: **Female *S. curvicornis* exhibit short period phenotype under constant darkness (<24 h endogenous**

346 **circadian rhythm)**. (i) Double-plotted actogram showing the locomotor activity pattern of: (A) The average of all 4

347 individuals examined of *S. curvicornis*. (B) A representative individual randomly selected from the population. In a

348 double plotted actogram, each row represents locomotor activity (counts per 30 min) of two consecutive days and

349 the second is repeated such that it is always the beginning of the next row. The x-axis shows the time of day under

350 constant darkness expressed as circadian time (CT). (ii) Average of the locomotor activity patterns of the five days

351 observed into one. The y-axis represents activity over time and the x-axis represents CT (iii) Autocorrelation plots

352 used to determine the period (p), rhythm index (RI) and rhythm strength (RS). The oscillations indicate periodicity.

353   The asterisk at the third peak of the autocorrelation plot indicates the particular time point used for the determination

354   of the rhythm parameters.

355   **Figure 3: A summary of the variations in the circadian rhythm as observed in: A)** *Lasioglossum malachurum***,**

356   **B)** *Lasioglossum enatum* and *Lasioglossum ferreirii*. The circle represents the root of the flowchart, squares

357   represent nodes that branch off and rhombuses represent leaves. In total for malachurum, 5 distinct behaviors were

358   observed.

359   **Figure 4: *L. malachurum* exhibits a variety of circadian phenotypes under constant dark conditions**. (i)

360   Double-plotted actogram showing the locomotor activity pattern for 5 days of: (A) The average of all 98 individuals

361   examined and representatives for the following categories: (B) Bimodal Rhythmic (C)Weakly Rhythmic Bimodal,

362   (D) Unimodal Rhythmic, (E) Weakly Rhythmic Unimodal, (F) and Arrhythmic circadian behaviors. (ii) An average

363   activity plot for the five days of observation (iii) Autocorrelation plots used to determine the period (p), rhythm

364   index (RI) and rhythm strength (RS).

365   **Figure 5: Description of the circadian behaviors exhibited by *L. ferrerii* under constant dark conditions.** (i)

366   Double-plotted actogram of the locomotor activity from the five-day observational period for: **A)** All 22 individuals

367   from the data set averaged out into one representative individual. **B)** A representative individual out of the 11 from

368   the category Strongly Rhythmic. **C)** A representative individual out of the 11 from the category Noisy Rhythmic.

369   (ii)An average activity plot for the five days of observation (iii) Autocorrelation plots used to determine the period

370   (p), rhythm index (RI) and rhythm strength (RS).

371   **Figure 6: Description of the circadian behaviors exhibited by *L. enatum* under constant dark conditions.** (i)

372   Double-plotted actogram of the locomotor activity from the five-day observational period for: **A)** All 8 individuals

373   from the data set averaged out into one representative individual. **B)** The only individual from the category Strongly

374   Rhythmic. **C)** A representative individual out of the 2 from the category Noisy Rhythmic. **D)** A representative

375   individual out of the 5 from the Arrhythmic category. (ii)An average activity plot for the five days of observation

376   (iii) Autocorrelation plots used to determine the period (p), rhythm index (RI) and rhythm strength (RS).

377   **Figure 7: Summary of descriptive and inferential statistics. A)** Number of circadian categories observed by

378   species. **B)** Box plot illustrating the difference in average locomotor activity between species. *S.*

14

379 *curvicornis* has a minimum of 8.200, 25% percentile of 8.900, mean of 13.63, 75% percentile of 20.28 and

380 a maximum of 22.90. *L. ferrerii* has a minimum of 0.7000, 25% percentile of 2.675, mean of 4.950, 75%

381 percentile of 7.100 and a maximum of 11.20. *L.enatum* has a minimum of 1.000, 25% percentile of 2.875,

382 mean of 8.113, 75% percentile of 6.650 and a maximum of 36.00. *L, malachurum* has a minimum of 6.700,

383 25% percentile of 10.35, mean of 8.201, 75% percentile of 10.35 and a maximum of 26.30. There was only

384 a statistical difference between *L. ferrerii* and *L. malachurum* with a p-value of 0.0016, DF of 61.66 and t

385 of 3.867. **C)** Box plot illustrating the difference in circadian period between species. *S.curvicornis* has a

386 minimum of 22.20, 25% percentile of 22.35, mean of 22.75, 75% percentile of 23.10 and a maximum of

387 23.20. *L. ferrerii* has a minimum of 21.80, 25% percentile of 21.95, mean of 22.69, 75% percentile of 23.20

388 and a maximum of 24.200. *L.enatum* has a minimum of 20.00, 25% percentile of 22.40, mean of 23.31,

389 75% percentile of 24.60 and a maximum of 25.50. *L, malachurum* has a minimum of 20.20, 25% percentile

390 of 23.50, mean of 24.00, 75% percentile of 24.50 and a maximum of 27.80. Both S. *curvicornis* and *L.*

391 *ferrerii* were significantly different from *L. malachurum* with p-values of; 0.0102 and <0.0001, DFs of;

392 5.652 and 52.94 and, t of; 5.179 and 6.565, respectively. **D)** Box plot illustrating rhythm strength among

393 species. *S. curvicornis* has a minimum of 3.000, 25% percentile of 3.250, mean of 4.000, 75% percentile of

394 4.500 and a maximum of 4.500. *L. ferrerii* has a minimum of 0.6000, 25% percentile of 1.700, mean of

395 2.691, 75% percentile of 4.125 and a maximum of 4.600. *L. enatum* has a minimum of -2.500, 25%

396 percentile -0.4250, mean of 0.2125, 75% percentile of 1.200 and a maximum of 2.200. *L, malachurum* has

397 a minimum of -2.100, 25% percentile of 0.7250, mean of 1.754, 75% percentile of 2.800 and a maximum

398 of 4.500. The solitary, *S. curivcornis*, and communal *L. ferrerii*, were significantly different from the

399 eusocial species, but not each other. Likewise, *L. enatum* and *L. malachurum* were not significantly

400 different.*S. curvicornis* vs. *L. enatum*   ; p-value of 0.0014, df of 7.932 and t of 6.226. *S. curvicornis*

401 vs. *L. malachurum*; p-value of 0.0177, df 4.04 of and t of 5.895. *L. ferrerii* vs. *L. enatum*; p-value of

402 0.0056, df of 17.09 and t of 3.982. *L. ferrerii* vs. *L. malachurum*; p-value of 0.0259, df of 33.42 and t of
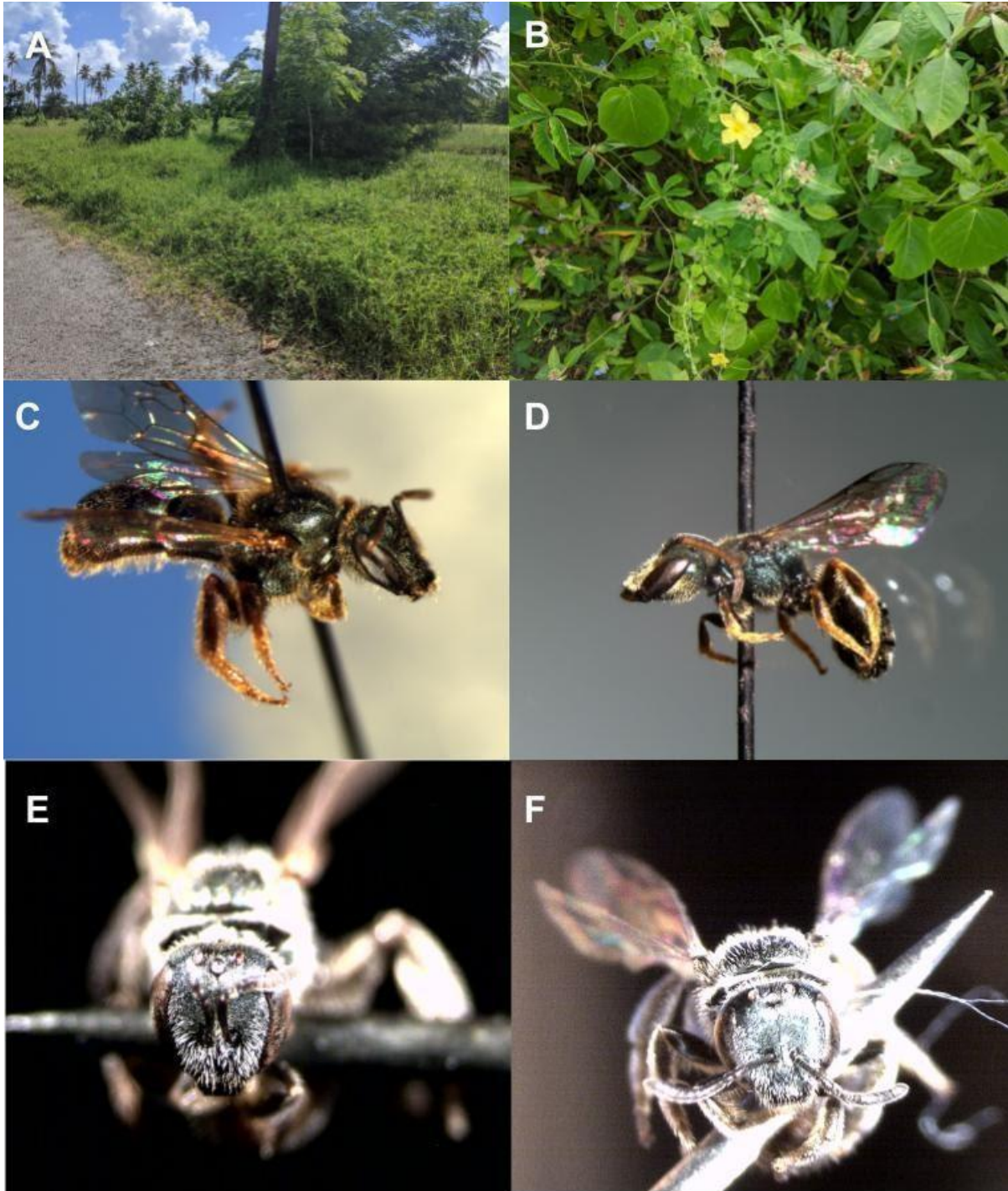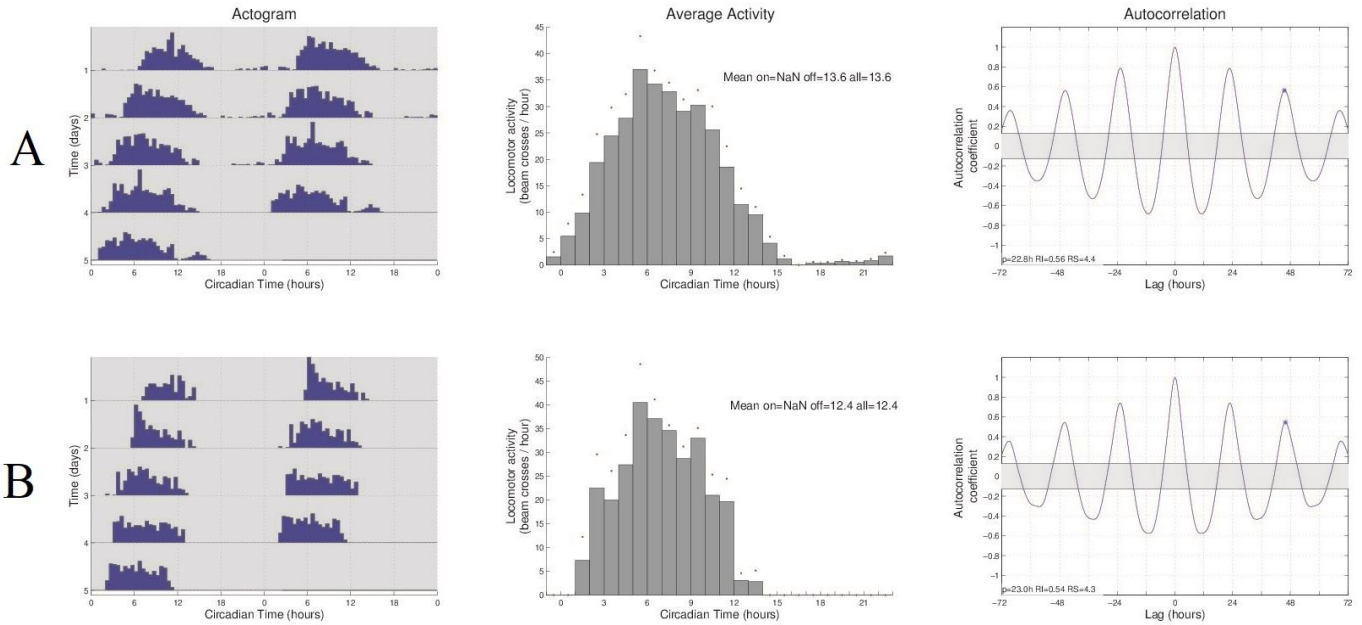
403 3.054.

404

405

15

406

407

408 **Tables and Figures:**

409
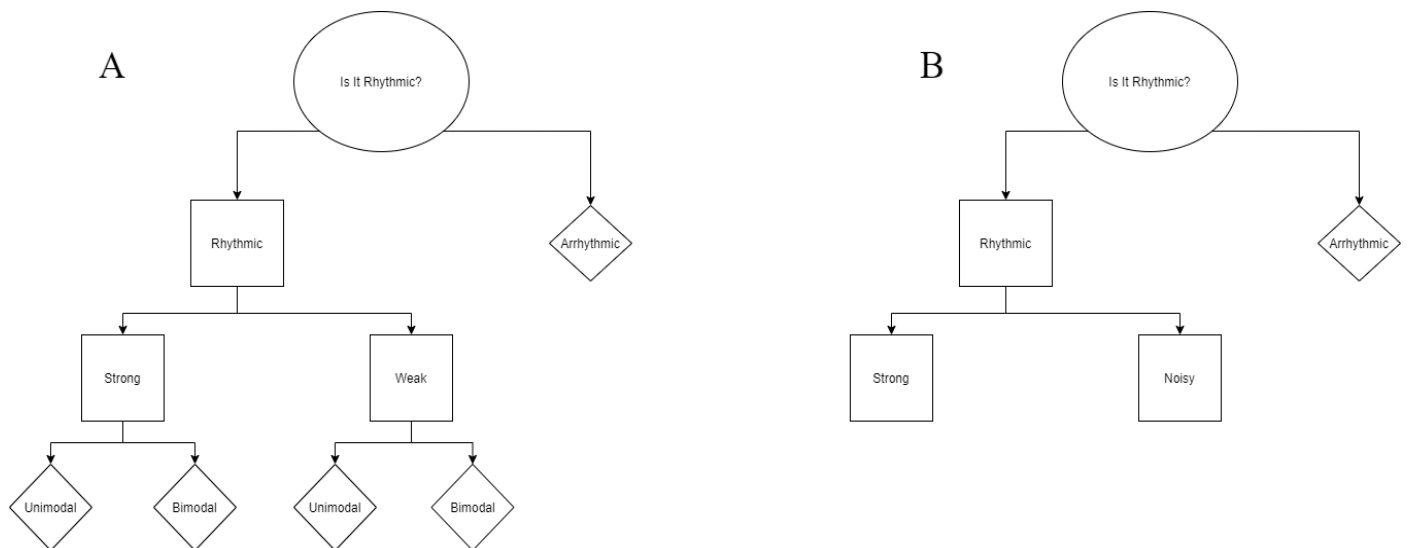
410 **Figure 1: Habitat (A-B) and Species Observed (C-F).**

411

412

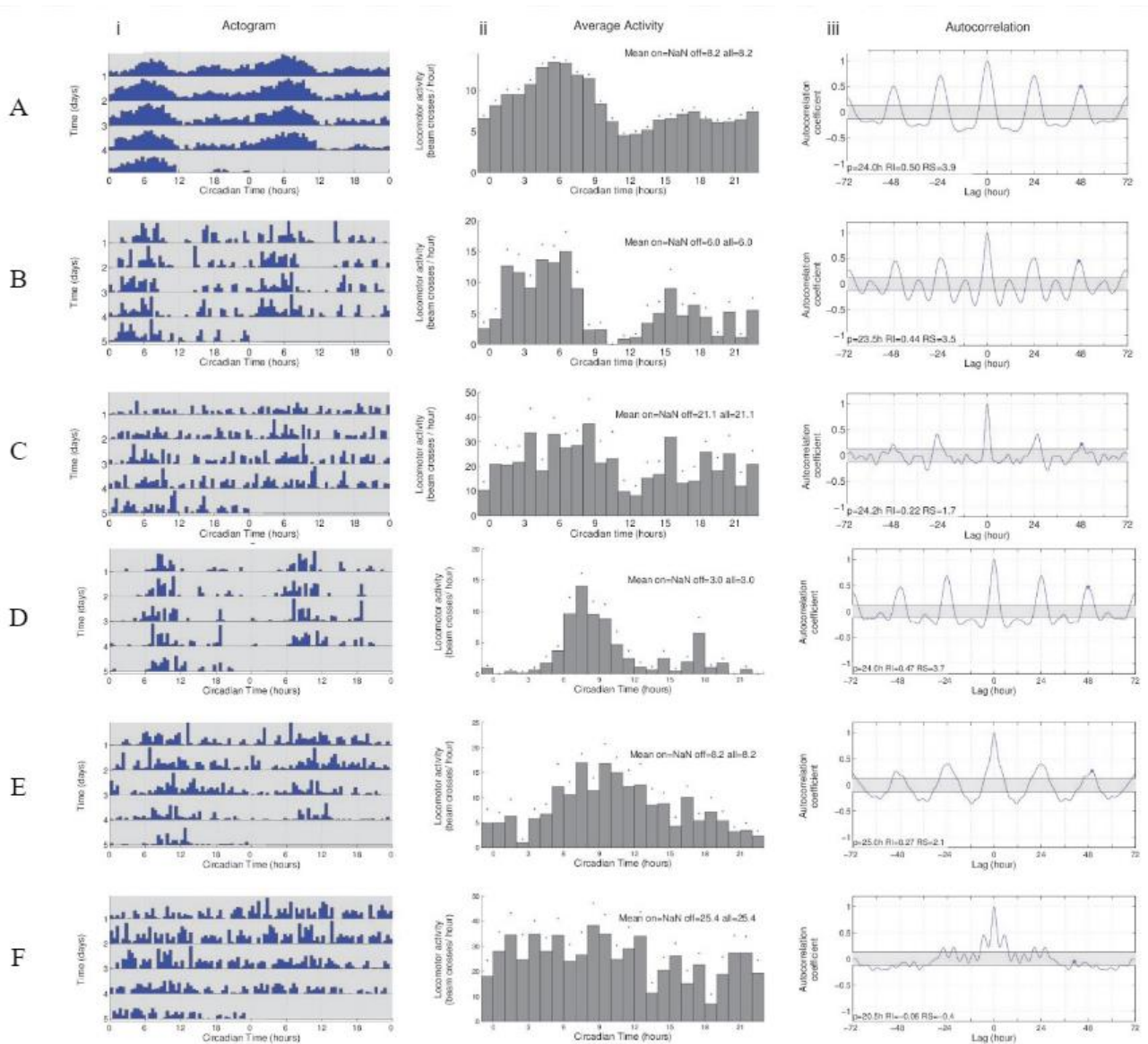413 **Figure 2**: **Female *S. curvicornis* exhibit short period phenotype under constant darkness (<24 h endogenous**
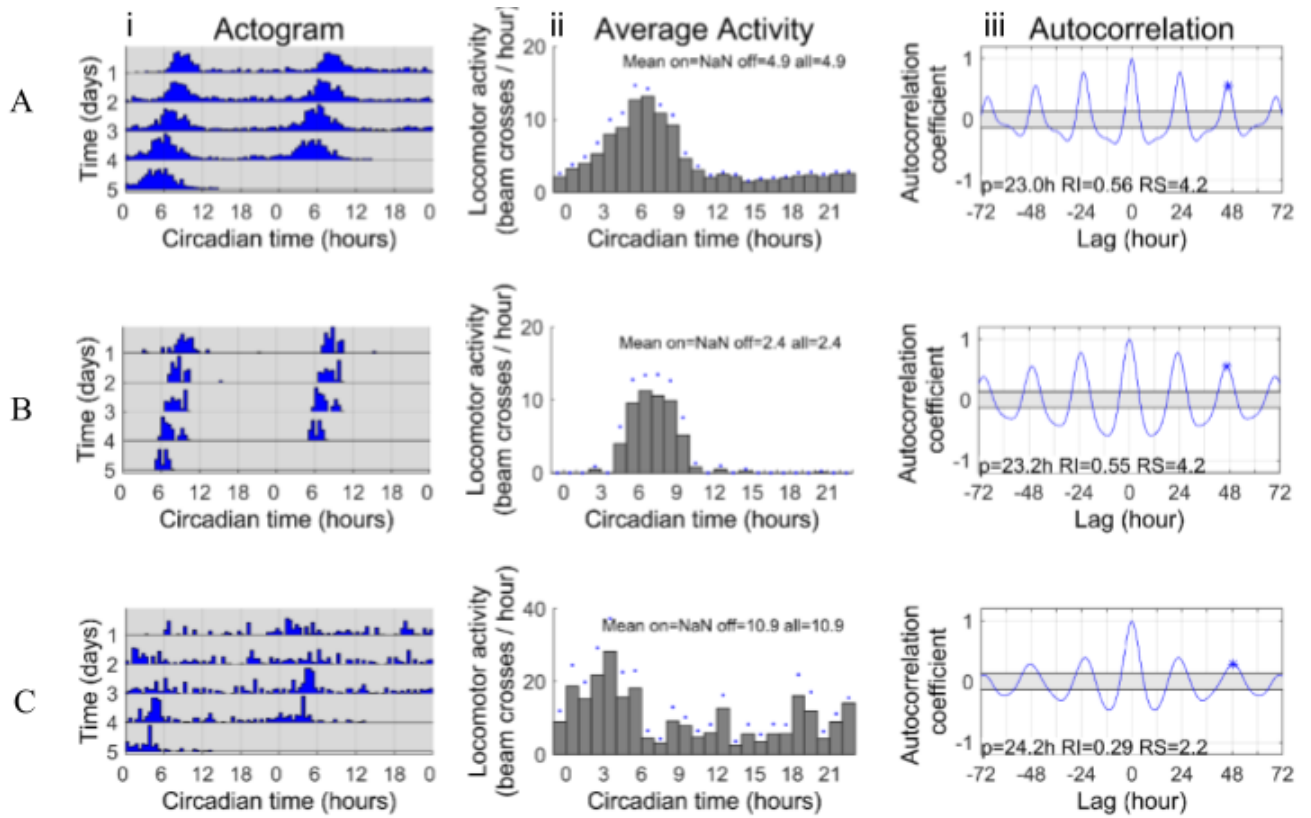
414 **circadian rhythm)**

415

416

17

417 **Figure 3: A summary of the variations in the circadian rhythm as observed in: A)** *Lasioglossum malachurum*,

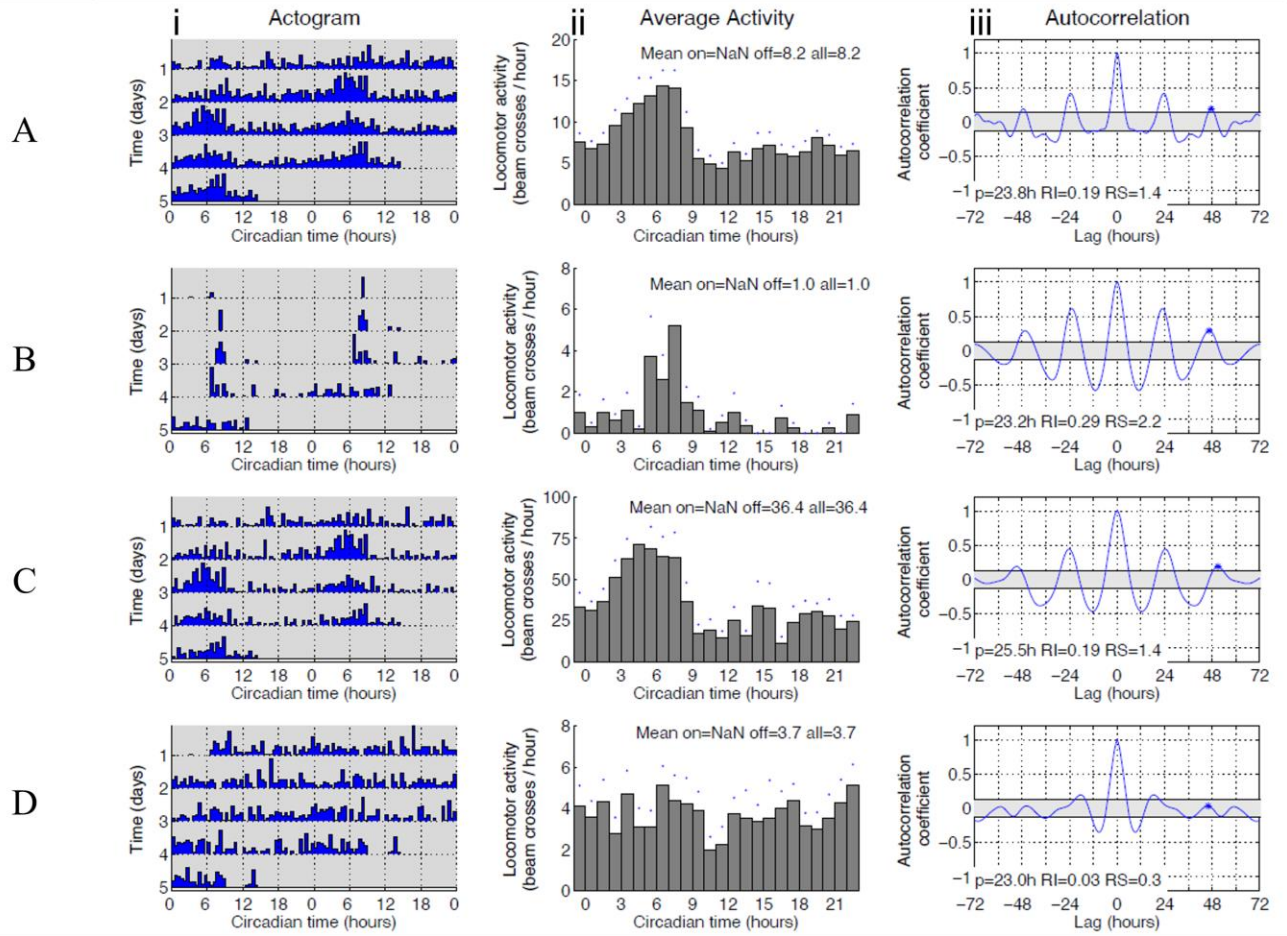418 **B)** *Lasioglossum enatum* and *Lasioglossum ferreirii*

419

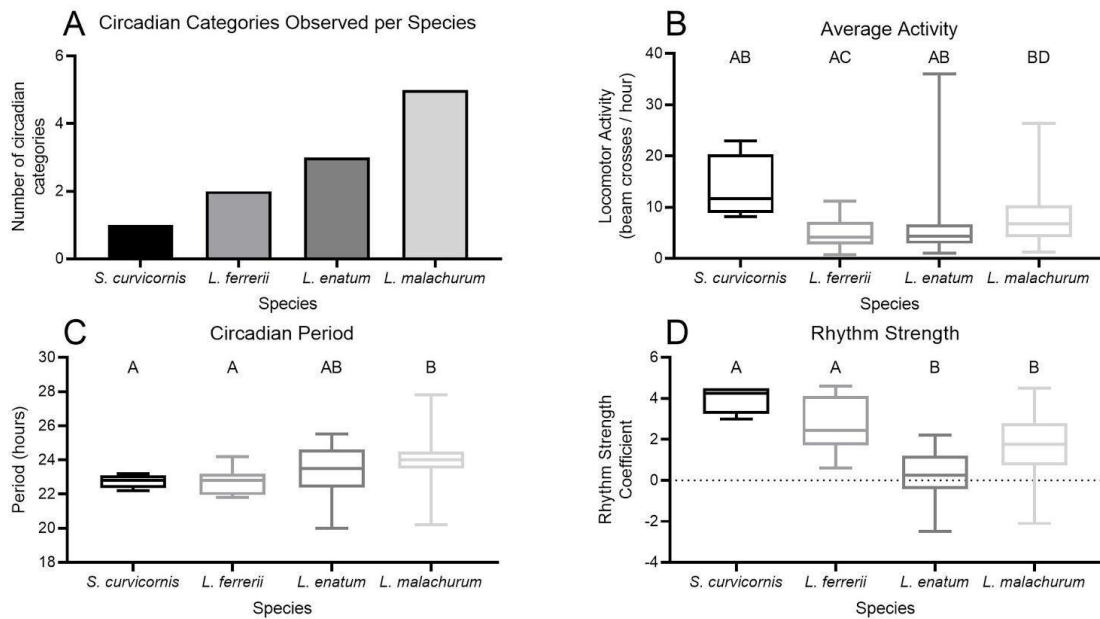420 **Figure 4: *L. malachurum* exhibits a variety of circadian phenotypes under constant dark conditions**.

18

Figure 5: Description of the circadian behaviors exhibited by *L. ferrerii* under constant dark conditions.

19

Figure 6: Description of the circadian behaviors exhibited by *L. enatum* under constant dark conditions.

Figure 7: **Summary of descriptive and inferential statistics**.

438   **Literature Cited:**

439   **Beer, K., & Helfrich-Förster, C. (2020).** Post-embryonic Development of the Circadian Clock Seems to Correlate

440   With Social Life Style in Bees. Frontiers in Cell and Developmental Biology, 8, 1325.

441   **https://doi.org/10.3389/fcell.2020.581323**

442   **Bloch, G., & Grozinger, C. M. (2011).** Social molecular pathways and the evolution of bee societies. Philosophical

443   Transactions of the Royal Society B: Biological Sciences, Vol. 366. https://doi.org/10.1098/rstb.2010.0346

444   **Bloch, G., Toma, D. P., & Robinson, G. E. (2001).** Behavioral rhythmicity, age, division of labor and period

445   expression in the honey bee brain. Journal of Biological Rhythms, 16(5), 444–456.

446   https://doi.org/10.1177/074873001129002123

447   **Danforth, B. N., Conway, L., & Ji, A. S. (2003**). Phylogeny of Eusocial Lasioglossum Reveals Multiple Losses of

448   Eusociality within a Primitively Eusocial Clade of Bees (Hymenoptera: Halictidae). Syst. Biol, 52(1), shrimp.

449   https://doi.org/10.1080/10635150390132687

450   **Delphia, C. M., & Gibbs, J. (2020**). New Island Records for Lasioglossum (Hymenoptera: Halictidae) from the

451   Virgin Islands, West Indies. Journal of the Kansas Entomological Society, 92(2). https://doi.org/10.2317/0022-8567-

452   92.2.479

453   **Dubowy, C., & Sehgal, A. (2017).** Circadian rhythms and sleep in Drosophila melanogaster. Genetics, 205(4),

454   1373–1397. https://doi.org/10.1534/genetics.115.185157

455   **Eickwort G.C. (1988).** Distribution patterns and biology of West Indian sweat bees (Hymenoptera: Halictidae). In

456   Liebherr J.K. (Ed.), Zoogeography of Caribbean Insects (pp. 232–253). Ithaca: Cornell University Press.

457   **Eickwort G.C., Eickwort J.M, Gordon J., Eickwort M.A. (1996)** Solitary behavior in a high-altitude population

458   of the social sweat bee Halictus rubicundus (Hymenoptera: Halictidae), Behav. Ecol. Sociobiol. 38, 227–233.

459   **Field, J. (1996)** Patterns of provisioning and iteroparity in a solitary halictine bee, Lasioglossum (Evylaeus)

460   fratellum (Perez), with notes on L. (E.) calceatum (Scop.) and L. (E.) villosulum (K.). Insect. Soc. 43, 167–182

461   **Field, J., Paxton, R.J., Soro, A., Bridge, C., 2010.** Cryptic plasticity underlies a major evolutionary transition.

462   Curr. Biol. 20, 2028–2031.

463   **Giannoni-Guzmán, M. A., Avalos, A., Perez, J. M., Loperena, E. J. O., Kayým, M., Medina, J. A., … Agosto-**

464   **Rivera, J. L. (2014).** Measuring individual locomotor rhythms in honey bees, paper wasps and other similar-sized

465   insects. Journal of Experimental Biology, 217(8), 1307–1315. https://doi.org/10.1242/jeb.096180

466   **Giannoni-Guzmán, M. A., Aleman-Rios, J., Melendez Moreno, A. M., Diaz Hernandez, G., Perez, M.,**

467   **Loubriel, D., … Agosto-Rivera, J. L. (2020).** The Role of Temperature on the Development of Circadian Rhythms

468   in Honey Bee Workers Authors. BioRxiv, 2020.08.17.254557. https://doi.org/10.1101/2020.08.17.254557

469   **Gibbs, J. (2018).** Bees of the genus Lasioglossum (Hymenoptera: Halictidae) from Greater Puerto Rico, West

470   Indies. European Journal of Taxonomy, 0(400). Retrieved from

471   https://europeanjournaloftaxonomy.eu/index.php/ejt/article/view/528/1183

472   **Gibbs, J., Brady, S. G., Kanda, K., & Danforth, B. N. (2012**). Phylogeny of halictine bees supports a shared

473   origin of eusociality for Halictus and Lasioglossum (Apoidea: Anthophila: Halictidae). Molecular Phylogenetics and

474   Evolution, 65(3). https://doi.org/10.1016/j.ympev.2012.08.013

475   **Gonzalez, V. H., Pascual, C., Burrows, S., Çakmak, I., & Barthell, J. F.** (2014). Pollen collecting behavior of

476   Systropha planidens Giraud, 1861 (Hymenoptera: Halictidae) in Turkey. Pan-Pacific Entomologist, 90(4), 226–230.

477   https://doi.org/10.3956/2014-90.4.226

478   **Grozdanic´, S., and Z. Mucˇalica. 1966**. Observations on the solitary bees Systropha planidens Gir., and Systropha

479   curvicornis Scop. Bulletin de Muse´um d'Historie Naturelle de Belgrade 21b:133–138 [In Serbo-Croatian, with

480   German summary].

481   **Helm, B., & Visser, M. E. (2010).** Heritable circadian period length in a wild bird population. Proceedings of the

482   Royal Society B: Biological Sciences, 277(1698). https://doi.org/10.1098/rspb.2010.0871

483   **Jeanson, R., Clark, R., Holbrook, C., Bertram, S., Fewell, J., & Kukuk, P. (2008).** Division of labour and

484   socially induced changes in response thresholds in associations of solitary halictine bees.

485   https://doi.org/10.1016/j.anbehav.2008.04.007ï

486   **Jud, C., Schmutz, I., Hampp, G., Oster, H., & Albrecht, U. (2005**). A guideline for analyzing circadian wheel-

487   running behavior in rodents under different lighting conditions. Biological Procedures Online, 7(1), 101–116.

488   https://doi.org/10.1251/bpo109

489   **Levine, J. D., Funes, P., Dowse, H. B., & Hall, J. C. (2002).** Signal analysis of behavioral and molecular cycles.

490   BMC Neuroscience, 3. https://doi.org/10.1186/1471-2202-3-1

491   **Mistlberger, R. E., & Skene, D. J. (2004).** Social influences on mammalian circadian rhythms: Animal and human

492   studies. Biological Reviews of the Cambridge Philosophical Society, Vol. 79, pp. 533–556.

493   https://doi.org/10.1017/S1464793103006353

494   **Moore, D., Angel, J. E., Cheeseman, I. M., Fahrbach, S. E., & Robinson, G. E. (1998).** Timekeeping in the

495   honey bee colony: Integration of circadian rhythms and division of labor. Behavioral Ecology and Sociobiology,

496   43(3), 147–160. https://doi.org/10.1007/s002650050476

497   **Ortiz-Alvarado, Carlos A,. Ambrose, Alexandria F., Cordero-Martinez, Claudia S., Silva-Echeandia,**

498   **Sebastian A,. Petanidou, Theodora, Tscheulin Thomas, Gonzalez, Victor H., Giray, Tugrul, Agosto-Rivera,**

499   **Jose A., Barthell, John F.** (*in rev*) Daily foraging activity and circadian rhythms of a guild of three large carpenter

500   bee species in Chasteberry (Vitex agnus-castus L.) on a Greek island (Lesvos).

501   **Patiny, S., & Michez, D. (2007).** New insights on the distribution and floral choices of Systropha Illiger, 1806 in

502   Africa (Hymenoptera, Apoidea), with description of a new species from Sudan. Zootaxa, (1461), 59–68.

503   https://doi.org/10.11646/zootaxa.1461.1.6

504   **Patiny, S., Michez, D., & Danforth, B. N. (2008).** Phylogenetic relationships and host-plant evolution within the

505   basal clade of Halictidae (Hymenoptera, Apoidea). Cladistics, 24(3), 255–269. https://doi.org/10.1111/j.1096-

506   0031.2007.00182.x

507   **Peters, R. S., Krogmann, L., Mayer, C., Donath, A., Gunkel, S., Meusemann, K., … Niehuis, O. (2017).**

508   Evolutionary History of the Hymenoptera. Current Biology, 27(7). https://doi.org/10.1016/j.cub.2017.01.027

509   **Radmacher, S., & Strohm, E. (2010).** Factors affecting offspring body size in the solitary bee Osmia bicornis

510   (Hymenoptera, Megachilidae) Factors affecting offspring body size in the solitary bee Osmia bicornis

511   (Hymenoptera, Megachilidae)*. Apidologie, 41, 169–177. https://doi.org/10.1051/apido/2009064ï

512   **Richards, M. H. (2000).** Evidence for geographic variation in colony social organization in an obligately social

513   sweat bee, Lasioglossum malachurum Kirby (Hymenoptera; Halictidae). Canadian Journal of Zoology, 78(7).

514   https://doi.org/10.1139/z00-064

515   **Richards, M. H., von Wettberg, E. J. & Rutgers, A. C. (2003)** A novel social polymorphism in a primitively

516   eusocial bee. Proc. Natl Acad. Sci. USA 100, 7175–7180. doi:10.1073/pnas.1030738100

517   **Roenneberg, T., Wirz-Justice, A., & Merrow, M. (2003).** Life between Clocks: Daily Temporal Patterns of

518   Human Chronotypes. Journal of Biological Rhythms, 18(1). https://doi.org/10.1177/0748730402239679

519   **Schwarz, M. P., Richards, M. H., & Danforth, B. N. (2006)**. Changing Paradigms in Insect Social Evolution:

520   Insights from Halictine and Allodapine Bees. https://doi.org/10.1146/annurev.ento.51.110104.150950

521   **Steitz, I., Kingwell, C., Paxton, R. J., & Ayasse, M. (2018**). Evolution of Caste-Specific Chemical Profiles in

522   Halictid Bees. Journal of Chemical Ecology, 44(9), 827–837. https://doi.org/10.1007/s10886-018-0991-8

523   **Toth, A. L., & Rehan, S. M. (2017).** Molecular Evolution of Insect Sociality: An Eco-Evo-Devo Perspective.

524   Annual Review of Entomology, 62(1). https://doi.org/10.1146/annurev-ento-031616-035601

525   **VanderPlas, J. T. (2017).** Understanding the lomb-scargle periodogram. ArXiv, Vol. 236, p. 16.

526   **https://doi.org/10.3847/1538-4365/aab766**

527   **Westrich, P. (1989).** Die Wildbienen Baden-Wu¨rttembergs. Ulmer Verlag, Stuttgart, Germany. 972 pp

528   **Wyman, L. M., & Richards, M. H. (2003).** Colony social organization of Lasioglossum malachurum Kirby

529   (Hymenoptera, Halictidae) in southern Greece. Insectes Sociaux, 50(3). https://doi.org/10.1007/s00040-003-0647-7