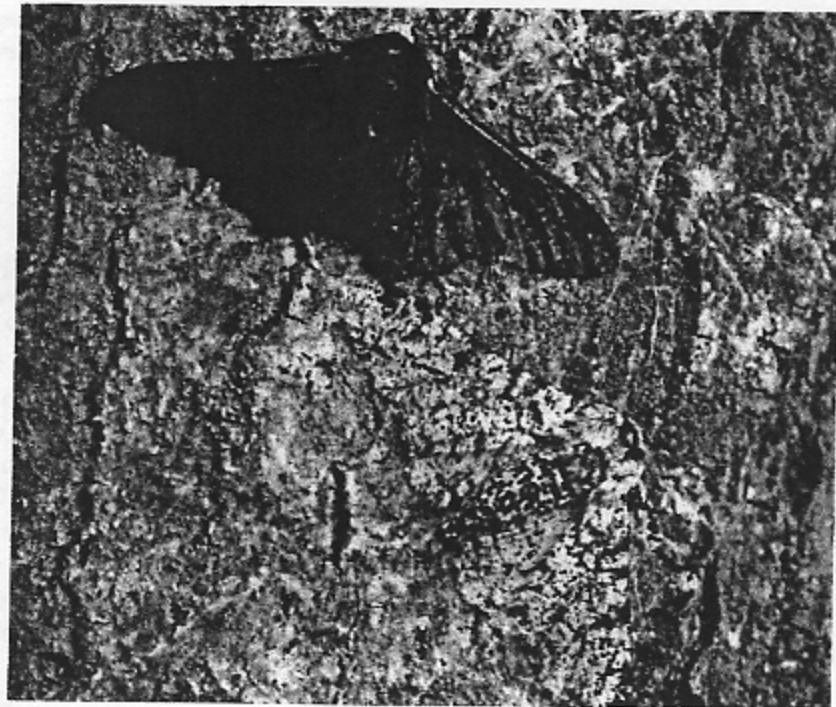


2009

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between May 2001 and August 2008 in Cambridge, UK

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ABSTRACT:

I look at existing knowledge of the resting sites of the Peppered Moth, *Biston betularia*, drawing on new data to present a more accurate and complete model of these locations. I relate the new information to key criticisms regarding the peppered moth case with respect to industrial melanism, differential selection pressures and natural selection. I extend existing ideas to propose a new theory on the variability in frequency of the morphs on different tree species, along with a new hypothesis on the frequency variation of the different colour morphs.

Key words: peppered moth, *Biston betularia*, carbonaria, typica, insularia, frequency dependent selection, industrial melanism, natural selection.

INTRODUCTION

Background:

The peppered moth, *Biston betularia*, is a small night flying species of univoltine moth with a wide distribution across the Northern Hemisphere, including North America, the United Kingdom, Holland and Scandinavia (Douwes *et al.* 1973). The species comes in a number of different cryptic forms. These range from the white-bodied black-speckled f. *typica* to the melanic f. *carbonaria*. Intermediate colourations exist as f. *insularia*, of which there are three morphs between f. *typica* and f. *carbonaria*. These intermediate forms were first described by Lemche (1931), though there have been many subsequent papers on the genetics of all the forms. (Lees 1968, Clark and Sheppard 1964, Steward 1977). The different phenotypes of the moths are the result of ‘- multiple alleles at a single locus that approximate a dominance hierarchy -’ (Grant 1999), placing f. *carbonaria* as the dominant, with f. *typica* as recessive (Lees and Creed 1977). These morphs of the moth appear at varying ratios in populations differing in location and time and thus have been extensively studied, especially in their relation to being one of the best active examples of Darwinian natural selection.

Breeding cycle:

A key requisite for anyone seeking to comprehensively grasp the peppered moth story is a detailed understanding of its lifecycle, only recently fully explored after extensive years of research by dedicated lepidopterists (Mikkola 1984, Liebert and Brakefield 1987, Majerus 1989). One of the most argued components of the case are the exact resting sites used by the moths when not on the wing, which this paper critically examines.

The life cycle of the peppered moth is relatively simple. The species has one brood per year, the primary larval instar hatches from eggs laid between May and August. These then go through a number of instar stages, feeding off the leaves in the trees in which they were laid. During this time the larvae can hang off the trees by silk threads, to quickly change position and to avoid predation. This may result in dispersal to other trees, for example by wind. They then drop from the trees into the leaf litter and pupate. The pupae over-winter in the soil, where they undergo the metamorphic changes necessary to eclose as imagines between late April and August. After hatching in the late afternoon the moths climb up the nearest tree, most likely *not* up into the higher canopy, as the twigs do not allow the moth to effectively utilise their ‘patch clamp’ behaviour resulting in reduced crypsis (Kettlewell 1955b). It is at this stage that the males and the females behave differently. The female will find a suitable branch in the tree, from which to release pheromones to attract the male (on the first night). She will not fly until having mated. On finding a male, they will start copulating during

the night, and stay together all the next day. After fertilisation, (the second night after hatching) she will go on her dispersal flight to find a suitable site to lay the eggs. This site is usually at the apex of a branch, in the cracks of the bark, if possible around lichens. After depositing her eggs, the female will not fly again, but instead will walk around the same tree for four to five days before dying. The males, however, fly every night, mating with as many females as possible.

The 'Classic' Story:

Evolutionary biologists have debated the story of the peppered moth since Kettlewell and Tinbergen's pilot study in 1955. Before this, it was already recognised that there was a frequency shift in the abundance of *f. carbonaria*, as after the discovery of the morph in 1848 it rapidly spread. This was especially evident in industrial regions, such as Manchester, which had 98% *f. carbonaria* in 1895. In 1896, the lepidopterist J. W. Tutt proposed that differential bird predation on the melanic *f. carbonaria* and *f. typica* caused their frequencies to vary depending on how much their habitats had been affected by industrial pollution. Extensive experimentation by Kettlewell in the 1950's (Kettlewell 1955 and 1959) at multiple locations across the UK provided evidence for this frequency change being due to industrial melanism. Almost all evolutionary biologists now voice this theory. During the industrial revolution, huge volumes of soot and sulphur dioxide, along with countless other pollutants were released into the air around the big industrial cities. The sulphur dioxide had a detrimental effect on the surrounding environment, causing the less tolerant plants and photosymbionts (the algae in the lichens) to be killed. The soot significantly darkened the bark of surrounding trees. This affected the cryptic colouration of the morphs, resulting in differential bird predation leading to a frequency distribution difference between forms, depending on whether they were in a polluted or unpolluted habitat. However, the Clean Air Act of 1956 caused a shift in the levels of pollutants that were allowed into the atmosphere. From the mid to the end of the 20th Century, the decrease in *f. carbonaria* correlated with the increase in air quality (Grant *et al.* 1998).

Modern opinion divided:

Further analysis of Kettlewell's experimental procedures led critics to suggest that the evidence was flawed, primarily due to perceived problems in the sampling technique used. The result was an explosion of papers in recent years refuting Kettlewell's research (Sargent *et al.* 1998, Rudge 1999, Wells 1999, Allchin 2001). Judith Hooper went as far as to insult Kettlewell in her controversial book, *Of Moths and Men*, by describing him as having "committed scientific fraud." She goes on to describe the case as "a fascinating psychological dissection of the ambitious scientists who will ignore the truth for the sake of fame and recognition". There were claims that peppered moths never actually naturally rested on the trunks of trees and thus the predation experiments were incorrect (Coyne 1998, Sargent *et al.* 1998). Majerus himself admitted that the story was 'incomplete', but has regularly been misquoted by the creationists, who pounced on the apparent weakness of the case. Many of the journalists that have recently surfaced to write the misinformed reports in the case have very likely never seen a peppered moth in the wild. David Rudge (1999), a critic of the classical story, concisely quotes 'experiments in evolutionary biology are simply meaningless divorced from a background of ecological and life history data provided by strictly observational field studies'. We are now in a position in the debate where this has been reached. There are enough data available, and enough field studies have been carried out to accurately document the entire lifecycle of the peppered moth, allowing conclusive answers to be reached for the majority of the questions surrounding the case.

This paper will provide strong evidence for the final resting positions of the peppered moths during the day, drawing on a dataset of 177 individual moths collected over the past eight years.

Hypotheses and comparisons to be tested:

Resting position in tree:

- i. Resting sites of non-mating males vs. females.
 - a. Both sexes will have a preference for resting in the branches.
 - b. Resting sites on branches. There will be a preference for resting on the underside of branches.
- ii. Moths resting vertically will have a preference for the North side of the tree.
- iii. Resting height in tree:
 - a. There will not be a significant difference between forms in resting height in the tree.
 - b. There will not be a significant difference between male and female resting height in the tree

Tree species preference:

- i. The darker f. *carbonaria* will show a preference for tree species with darker bark.
- ii. The lighter f. *typica* will show a preference for tree species with lighter bark.

Date frequency distribution:

- i. f. *typica* will show an increase in relative abundance over the study period.
- ii. f. *carbonaria* will show a decrease in relative abundance over the study period.

MATERIALS and METHODS

Study site:

The study site is located in Soton, Cambridgeshire, on a piece of designated SSSI chalk land. Half of it is an old orchard. The sampled trees consist of: 14 apple, two pear, four wild plum, a cultivated plum, a goat sallow, four birch, an oak, two ash, two elder, 13 hawthorn, two blackthorn, a privet and a twisted willow. They ranged from 3.2m (apple 8) to 15.3m (ash 2) in height. These 48 trees are spread over an area of approximately one hectare. Surrounding the plot are agricultural land, deciduous hedges and small areas of deciduous woodland.

Data collection:

All of the data in this study has been collected by Professor Michael Majerus. Data collection involved climbing the trees within the study and sampling the trunk, branches and twigs for all *Biston betularia* morphs (f. *typica*, f. *carbonaria* and the three main distinctions of f. *insularia*). The lower trees were accessed by ladder to reach the canopy and the taller trees had climbing aids attached. This ensured that all heights in the tree could be sampled. Each moth found had its exact natural resting position documented, with readings taken for: date, form (typ, carb, ins 1/2/3), sex, tree found on (e.g. apple 4), height above ground level, vertical/horizontal (where horizontal is any position within 45° above or below the horizontal), resting orientation if vertical (N/S/E/W facing), part of tree found on (trunk/branch/twig), resting position on branch (top/mid/under), resting position on twig (bare/leafy/in leaves), resting position on trunk (exposed/in foliage).

Statistical analyses:

All data were checked for deviations from normality. A comparative analysis of the resting sites of the moths was tested using chi squared (as the points were counts data) to test for heterogeneity in the dataset (except when expected values were below five, in which case Fisher's exact test was used). The same tests were used for testing the resting height in the tree and the orientation preference. For the resting height analysis, the data was first made into a relative fraction, so that trees of different heights could be compared. Any trees that had less than five moths in were grouped together by species. Those tree species which did not have a large enough sampling set were excluded from this part of the analysis (twisted willow, privet, pear and plum). The data was then arcsine transformed, (as it was in the form of a percentage fraction). Normality was checked using an Anderson-Darling test. An ANOVA test was then used to test for variation between mean resting heights across the trees. Mating pairs were not included in the male vs. female comparisons, as it was uncertain as to whether the male or female most

likely influenced the choice of resting site (though probably female). Bonferroni corrections were used when testing subsets of the data to avoid rejecting the null hypothesis when it is true (a type I error). A Spearman's rank correlation coefficient test was used to test for the strength of any correlation found in the tree species preference comparison.

ANALYSIS

Resting position in tree:

Resting Sites of non-mating females vs. males:

A comparison of the resting sites of the non-mating moths in the sample set (153 subjects) revealed that there was a significant difference between choice of resting site (**Fig. 1**), with the majority of the moths resting on the branches ($\chi^2_{2, 0.05} = 36.58, N=154$). There was no significant difference in the sex ratio at each of the three sites ($\chi^2_{2, 0.05} = 4.40, N=154$). The twig resting position site on its own showed an apparent trend towards significantly more males than females ($\chi^2_{1, 0.05} = 3.55, N=21$), though after a Bonferroni correction, this was discarded. There was no significant difference in homogeneity between form ratios at each location (Fisher's Exact, FE: $P = 0.001, N=154$).

There was a significant preference for resting on the underside of branches (**Fig. 2**, FE: $P = 0.026, \chi^2_{2, 0.05} = 89.38, N=86$) but no overall significant difference in the sex ratio at each position (FE: $P = 0.076, N=86$). There was also no significant difference in the homogeneity of the different forms at each site (FE: $P = 0.0057, N=86$).

Vertical resting orientation:

Moths that were resting vertically, which accounted for 38.4% of those in the data set, showed a significant preference for the North side of the tree (**Fig. 3**, $\chi^2_{3, 0.05} = 53.05, N=68$). There was no difference in sex ratio for orientation (FE: $P = 0.012, N=68$). There was also no difference in homogeneity of forms at each of the four locations (FE: $P = 0.013, N=68$).

Resting height in tree:

The dataset showed a normal distribution (AD: $A^{*2} = 0.699, N=171$). An ANOVA test on the data showed that the sample groups (tree sets, see **Fig. 4a** caption) relative heights do not have equal means ($F_{16,170} = 6.109, P < 0.0001, N=171$), the mean resting height varies between tree species (**Fig. 4a and 4b**). There was not significant difference in resting heights between morphs in the sample set ($\chi^2_{2, 0.05} = 0.669, N=171$) or between sexes ($\chi^2_{1, 0.05} = 0.103, N=171$).

Tree species preference:

Tests on morph preference by tree species showed an inverse relationship between *f. carbonaria* and *f. typica* morphs (the two extreme forms). *F. typica* showed a preference for lighter barked trees (e.g. birch) and *f. carbonaria* showed a preference for darker colour trees, such as Plum (**Fig. 5**). Using a Spearman Rank correlation coefficient test, ranking trees from birch being lightest, and therefore 1, to plum being darkest, 6 (**Fig. 6**), *f. carbonaria* showed a large positive correlation between tree colour and frequency ($\rho = 0.933, N=15$). *F. typica* showed a strong negative correlation ($\rho = -1.07, N=118$) whilst *f. insularia* showed weak correlation ($\rho = -0.266, N=16$).

Date frequency distribution:

The frequency of *f. typica* showed an average gradual increase over each year (**Fig. 7a**). Conversely, *f. carbonaria* and *f. insularia* show an inversely fluctuating frequency distribution. *F. carbonaria* is decreasing over time, whilst the relative proportion of *f. insularia* remains constant over the eight years of the survey.

DISCUSSION

The results found in this study now allow a thoroughly conclusive answer to be given to the problem of where peppered moths naturally rest during the day. This will be presented here, and will also be used to counter some of the major recent criticisms of the peppered moth case (Wells 2007, Hooper 2002), whilst upholding the views of the vast majority of the most recent discussions and papers on the subject (Matzke 2007, Connor 2007, de Roode 2007). The majority of the claims made in Jonathon Wells' 2007 report, *Exhuming the peppered mummy*, are not supported by the data that has been analysed in this sample.

Resting sites:

The results obtained from this analysis allow a number of solid conclusions to be made. To begin with the data strongly supports the current hypothesis held of natural resting sites. The majority of the moths in the sample were found to rest in the branches (Fig. 1, 61%). However, a significant proportion of moths in the sample were also found to naturally rest on the trunks of the trees (25%), contradicting Wells suggestion that "the most embarrassing was that peppered moths in the wild don't normally rest on tree trunks" (Wells 2007). There was no significant difference between the relative numbers of the different forms at each site. This is due to the fact that there would be little difference between the average 'camouflage' offered by the three resting sites.

Of the moths that rested on branches, the overwhelming majority chose to reside on the underside of the branch (Fig. 2, 81%). Such positions are likely to maximise crypsis when the moths are resting during the day, when their main selective predators (the birds) are 'hunting'. The main predator of the moths (in terms of gross numbers eaten) is unknown, though it is thought likely to be bats. Contrary to Hooper's suggestion in *Of Moths and Men*, bats will have no selective effect on the frequencies of the different morphs within the population (Majerus 2008). Many well-designed experiments have shown that birds will selectively predate on the most conspicuous form of *B. betularia*. Thus the resting site of the moth is highly important. If the moths rest on a twig that is too thin, they are not able to exhibit their full clamp behaviour, in which they press their body against the surface they are resting on. This creates shadow effects and the possibility of the moth not being able to conceal its whole body plan onto the twig. Both of these will make the moth more conspicuous to avian predators. These are two possible reasons why such a small percentage of moths were found to be resting on twigs (13%), showing that in reality, the moths rarely rest high up in the canopy.

Those moths that were resting in a vertical position showed a significant preference for the North side of the tree (Fig. 3). There are two reasons for this: the moths have greatly reduced shadow effects (therefore increased crypsis) and the ambient temperature in the shade is more constant on the North side of the tree.

Resting height in tree:

The resting height of the moths showed that there was not any significant preference for either a particular height above ground level or for a particular relative height within the trees (Figures 4a and 4b), as indicated by the ANOVA result. Again this helps give evidence that the moths do *not* primarily rest in the upper canopy, but are more evenly distributed throughout the tree. This is counteracts Wells' (2007) suggestion that 'he (Majerus) couldn't see most of them because they were resting high in the upper branches of his trees. Those he could see from the ground represented only a tiny fraction of the total.'

Tree species preference:

The tree species encountered in this study showed a degree of difference in their bark colouration, from the pale coloured Birch (*Betula pendula*) to the dark Plum (*Prunus sp.*). In this comparison, Oak, Hawthorn, Ash and Apple were also looked at. Obviously within any tree species there will be variation in the colour of the bark, however, in general, these six species can be put in a rough bark colour order, from lightest to darkest (Fig. 6. Ash, Oak and Hawthorn are considered equal). The light silver birch has very fine black spots and also wider dark coloured bars (lenticels). This provides an excellent site for *f. typica* to be cryptically camouflaged against. However, in contrast the darker cherry bark provides

a much better surface against which *f. carbonaria* blends in. In between these two are the Ash, Oak, Hawthorn and Apple trees. The former three have similar bark colouration, with apple being also similar though often containing large patches of dark lichen, which would provide suitably cryptic resting sites for both *f. typica* and *f. carbonaria*. Therefore the results obtained in the experiment concur with this reasoning to some extent, including the Spearman's rank correlation test applied to the above arrangement in tree bark colour (Fig. 5). There are more *f. typica* found in Birch trees than in any of the others. The reason for this could be that the moths have a capacity to match themselves against the surface on which they are resting, by matching their own body scales to the surface reflectance, something Kettlewell (1955b) confirmed with his 'barrel experiments'. In contrast, *f. carbonaria* show their highest proportion from the Cherry tree, in which they are best cryptically hidden. Thus future studies would be wise to carefully look at the tree species used, as this appears to have an influence on the morph distribution.

Date frequency distribution:

The result achieved in this example is highly interesting. Very little prior work has been done looking at the frequencies of *f. insularia* within the population, as the majority of the work has focussed on the melanic *f. carbonaria*. As a result it was often overlooked in studies similar to this one. However, we can see that there is an apparent inverse fluctuation between the frequencies of *f. insularia* and *f. carbonaria* (Fig. 7). It would certainly be worthy of further study, as it could represent some form of negative frequency dependant selection (between the two less frequent morphs), with the presence of a conspicuous polymorphism in the population (*f. carbonaria*), maintained by apostatic selection. There is also an apparent 3-year cycle to this selection. This could be reflective of the time it takes for an avian predator to vary its search image of the forms. However, due to the small sample sizes of *f. insularia* and *f. carbonaria* per year, these hypotheses are entirely speculative and would require years of further data collection with a larger sampling effort before a confident result about this pattern could be given. The data given in a recent paper by Cook and Turner (2008) does not strongly support this hypothesis, however, again there are very low numbers of *f. insularia* in their data also.

For an unknown reason, there were more males than females found in the sampling, though I propose that this is due to a shorter lifespan of females than males after eclosion.

Conclusion:

This paper aims to provide the most conclusive report to date on the natural daytime resting sites of the peppered moth. The majority of the criticisms against the case have been addressed here, along with those reports that have presented evidence for the strength of the case. I now propose that a number of further studies be carried out, including more conclusive studies into the frequencies found between different tree species, and a closer look at the apparent frequency dependence between *f. insularia* and *f. carbonaria*. However, if *f. carbonaria* continues to decline at its current rate, it could be extinct from this population by 2018.

The vast majority of the reports that have been issued in the past two years on the subject all agree that the case of the peppered moth is a staple example of evolution by natural selection, a change driven by industrial melanism (Connor 2007, de Roode 2007, Cook and Turner 2008, Majerus 2008b). Grant (1999) reported, "In truth, we still don't know the natural hiding places of peppered moths." Fortunately now for this case, we do.

ACKNOWLEDGEMENTS

This paper would not have been possible without Professor Mike Majerus who spent eight years meticulously collecting the data for this study, published many papers on the topic and enthusiastically would answer any questions on the lifecycle of *Biston betularia*. To our deepest sadness, Professor Majerus passed away during the writing of this paper. Professor Majerus was a pinnacle player in laying the keystone for the more generalised understanding and teaching of evolution by Darwinian natural selection. He will be fondly remembered.

Also to Dr. Richard Hall for his excellent help in the statistics side of the project, to Dr. Remy Ware for her highly useful advice in my analysis and to Chris Jiggins for a concise general overview of the paper.

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RESULTS

Resting position in tree:

Resting Sites of non-mating females vs. males:



Figure 1. Resting sites of non-mating males vs. females. Branches: ($\chi^2_{2, 0.05} = 36.58$, $N=154$). Sex ratio: ($\chi^2_{2, 0.05} = 4.40$, $N=154$). Homogeneity of forms: (Fisher's Exact, FE: $P = 0.001$, $N=154$).

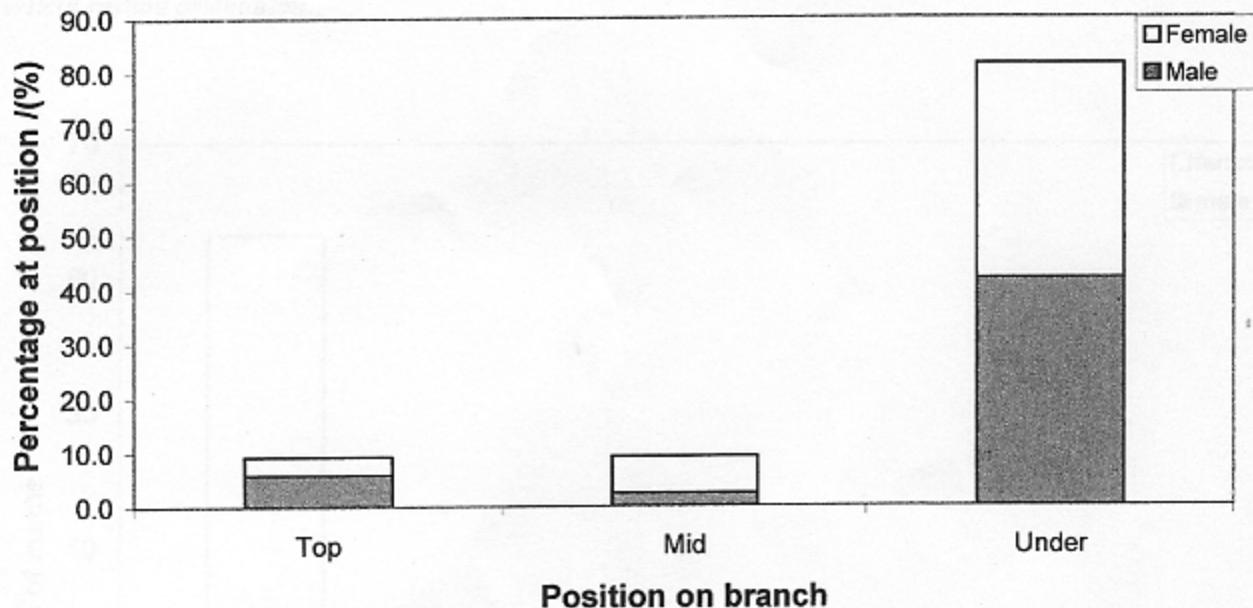


Figure 2. Resting sites of males and females on branches. Underside branch: (FE: $P = 0.026$, $\chi^2_{0.05} = 89.38$, $N=86$). Sex ratio: position (FE: $P = 0.076$, $N=86$). Homogeneity of forms: (FE: $P = 0.0057$, $N=86$).

Vertical resting orientation:

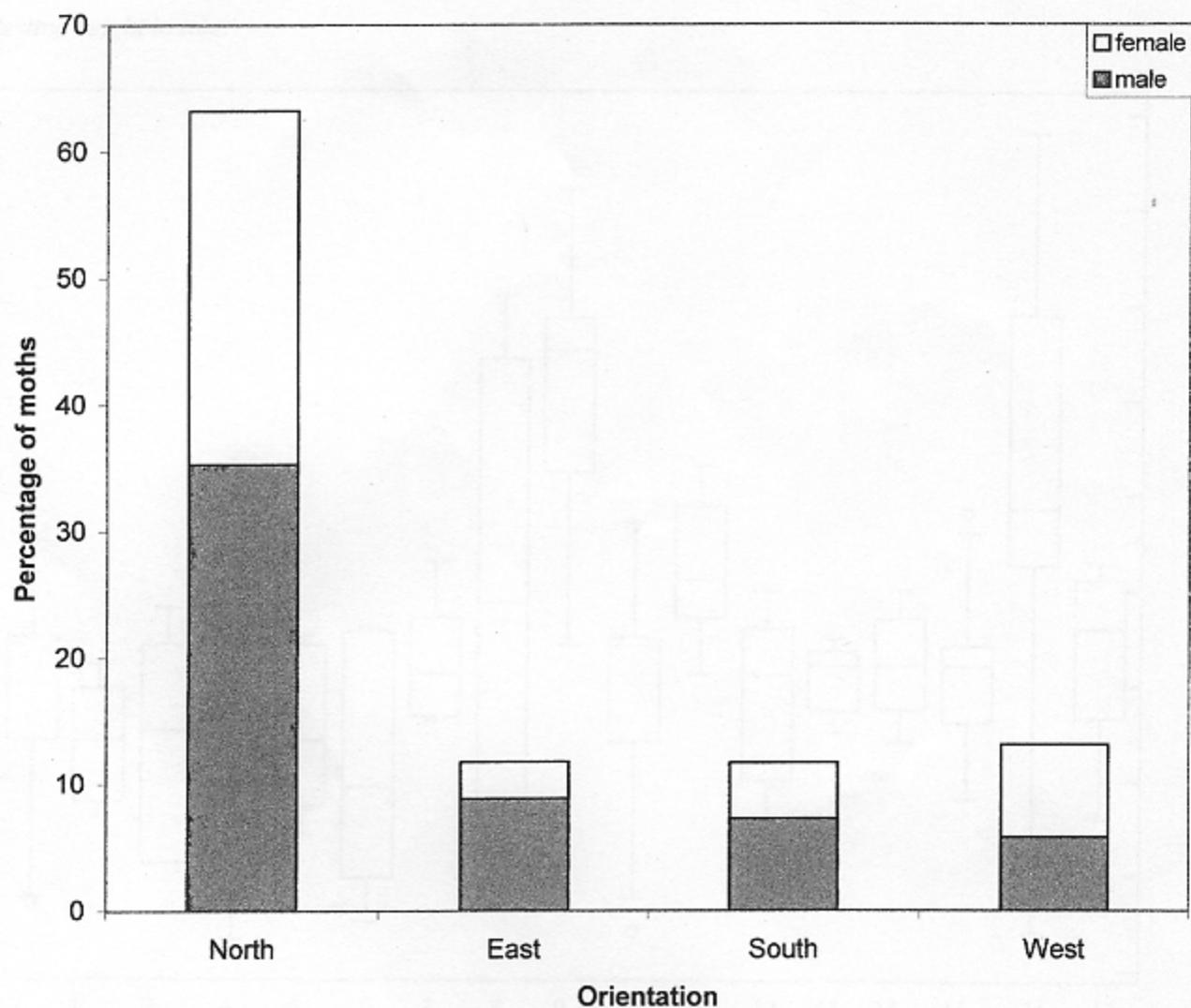
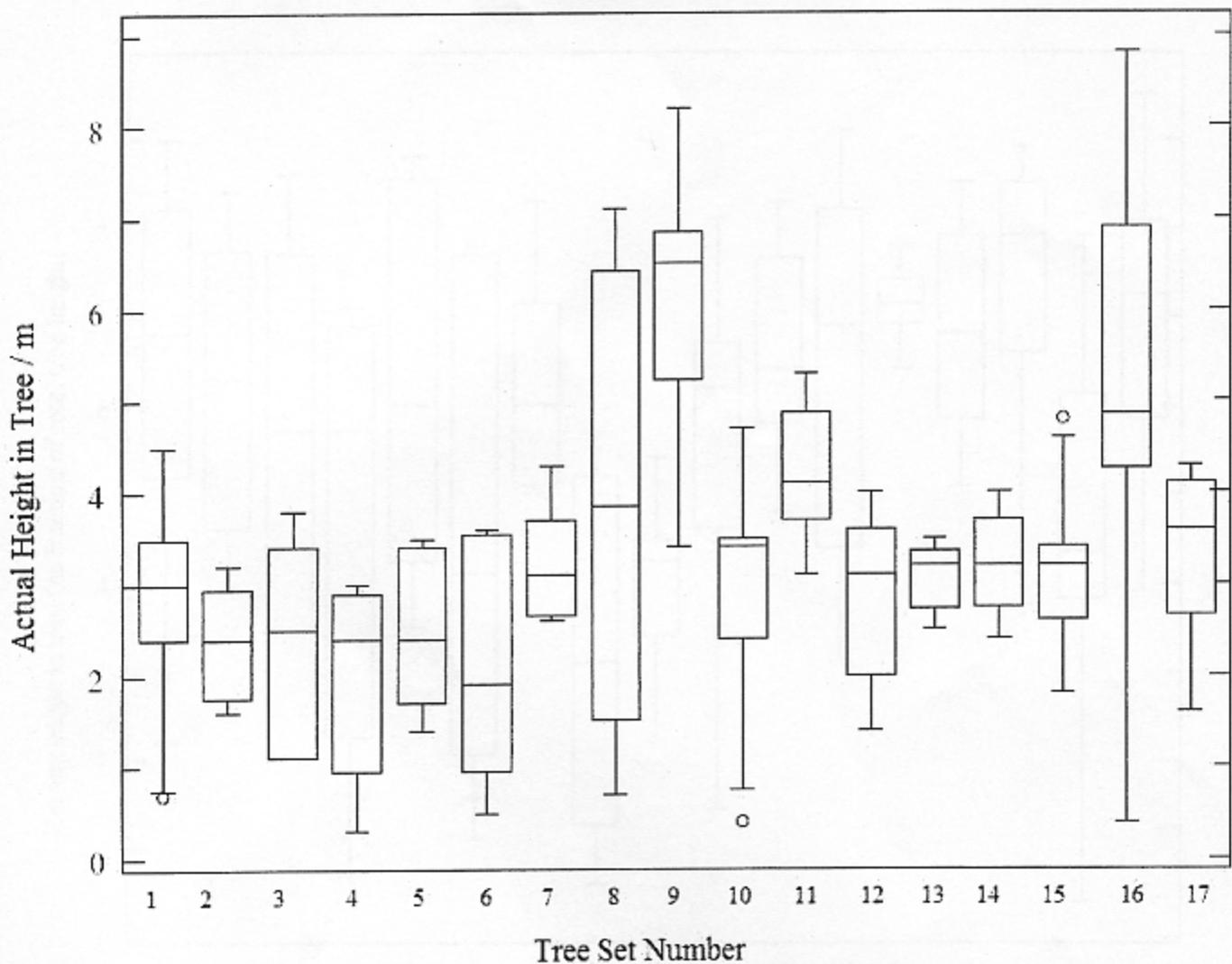


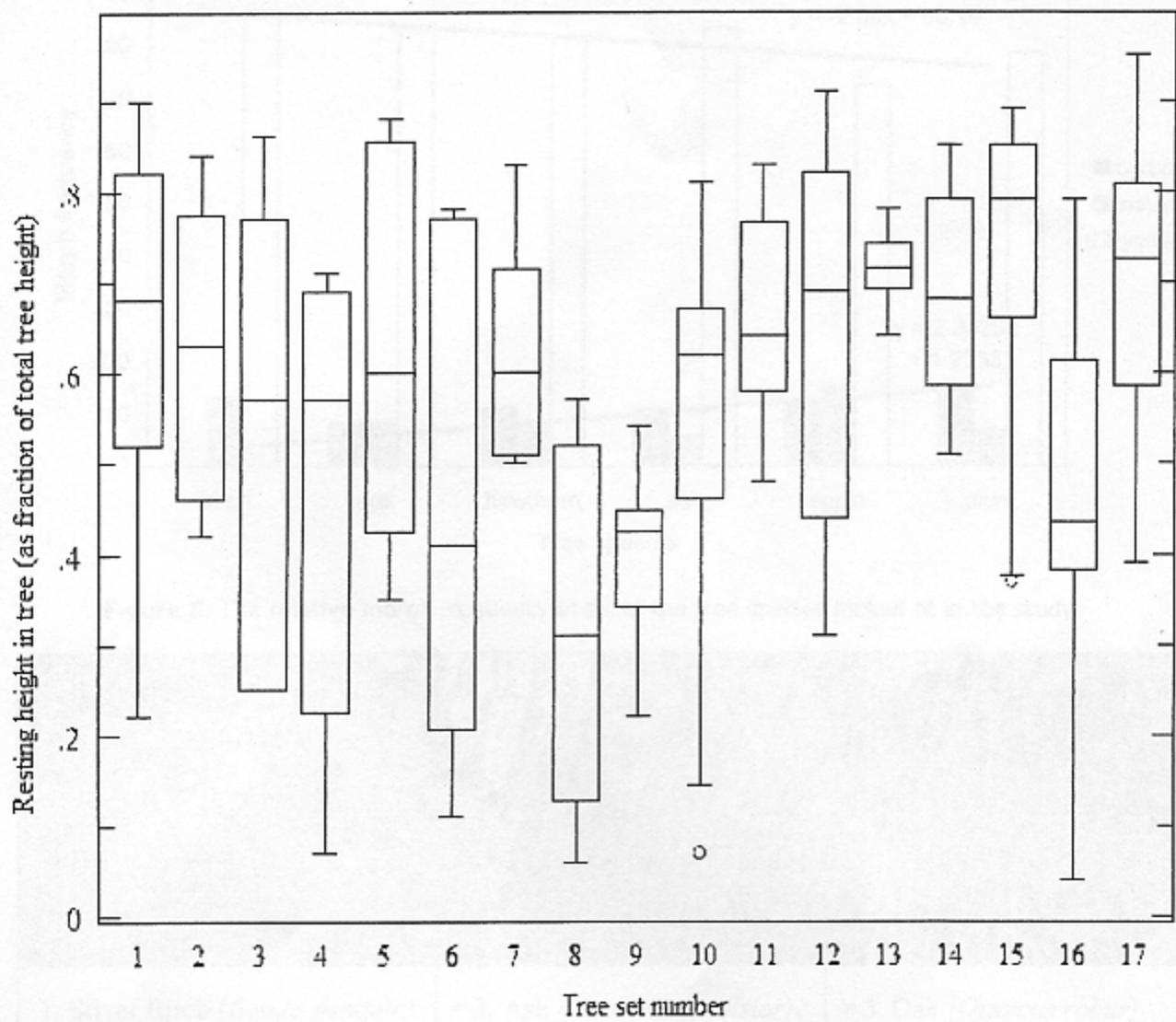
Figure 3. Orientation preference, with male:female ratio. North: ($\chi^2_{3, 0.05} = 53.05, N=68$). Sex ratio: (FE: $P = 0.012, N=68$). Homogeneity of forms: (FE: $P = 0.013, N=68$).

Resting height in tree:



Figures 4a. Boxplot of actual resting sites of *Biston betularia* in tree sets 1-17. Normal distribution:
(AD: $A^{*2} = 0.699$, $N=171$).

Tree sets refer to individual trees or where the individual count was <5, they are grouped by species:
1=apple, 2=apple4, 3=apple5, 4=apple7, 5=apple9, 6=apple10, 7=apple11, 8=ash1, 9=ash2,
10=beech, 11=beech3, 12=blackthorn, 13=elder, 14=goat sallow, 15=hawthorn, 16=oak, 17=wild
plum)



Figures 4b. Boxplot of relative (as fraction per tree height) resting sites of *Biston betularia* in tree sets 1-17. ANOVA on relative heights: ($F_{16,170} = 6.109, P < 0.0001, N=171$). Tree sets, see 4a.

Tree species preference:

Relative morph frequency by tree species

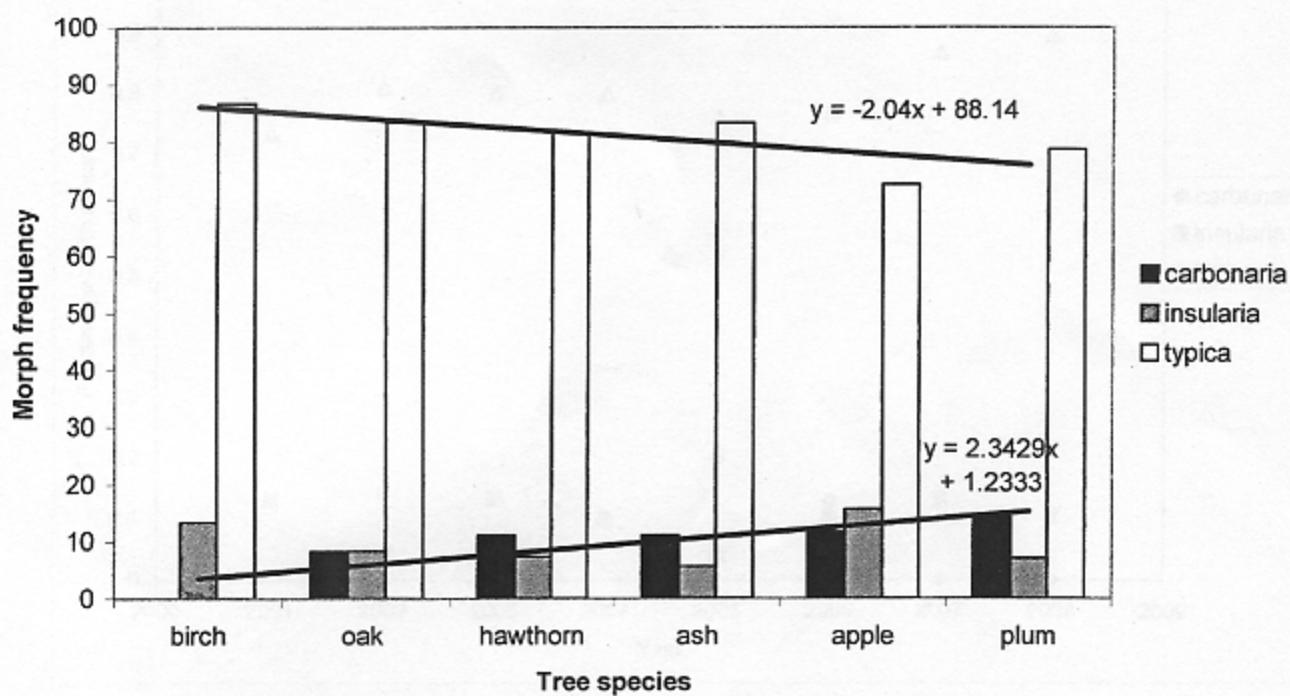


Figure 5. The relative morph frequency in six of the tree species looked at in the study.

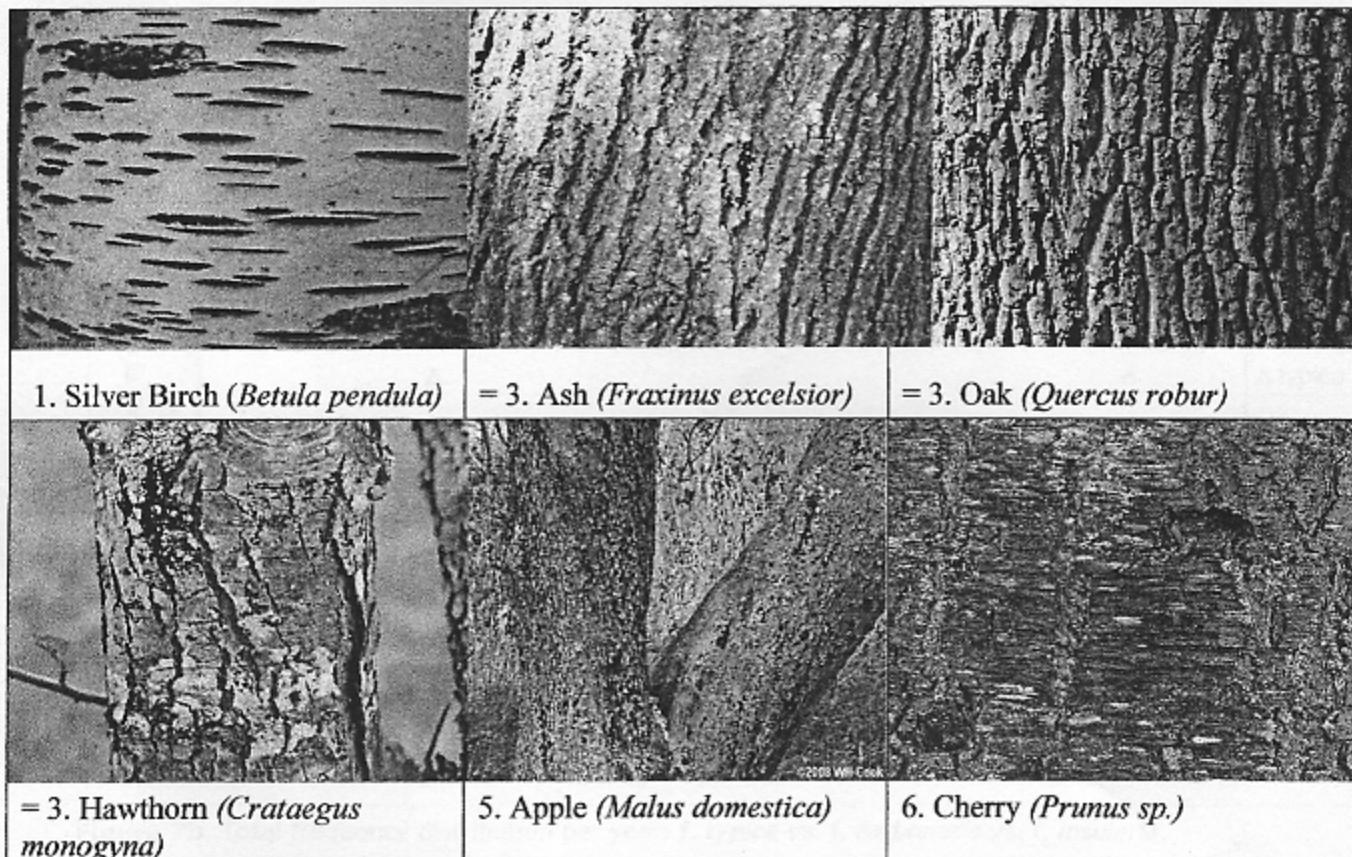


Figure 6. Typical bark colouration of the trees in the study.

Date frequency distribution:

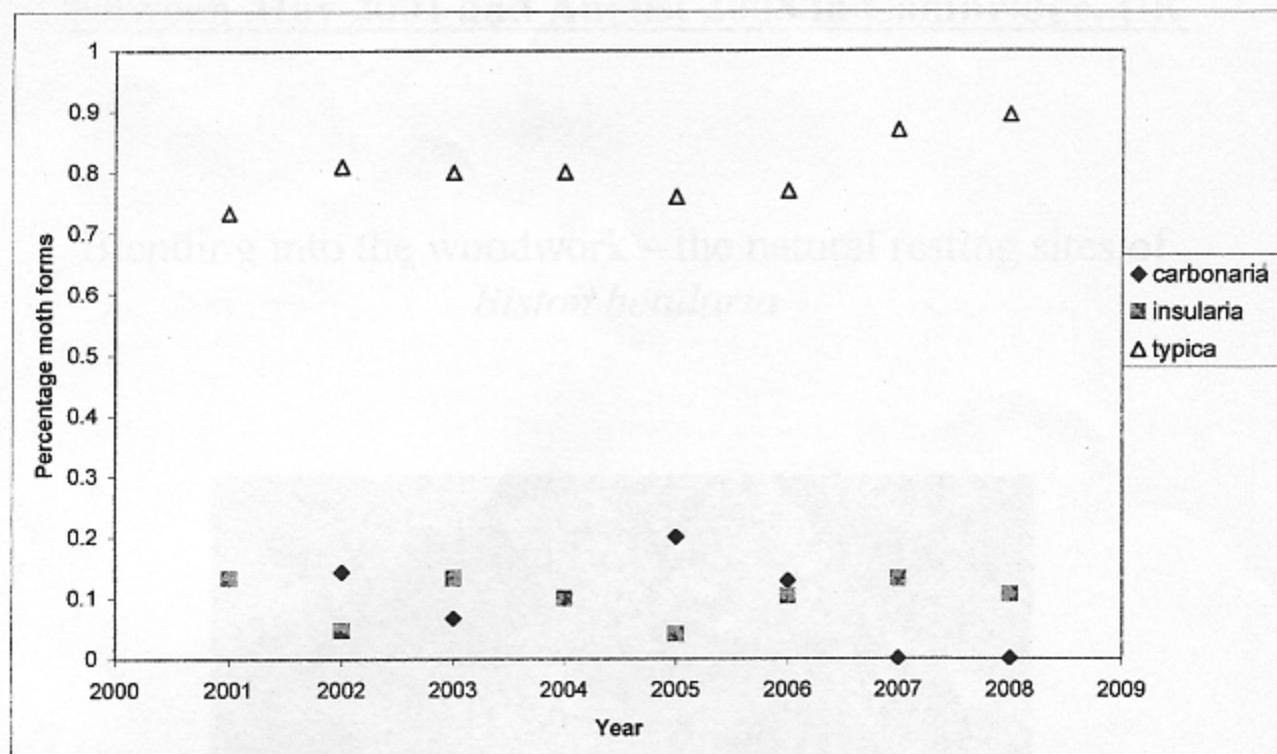


Figure 7a. Relative frequency distribution per year: f. typica vs. f. carbonaria vs. f. insularia (there was insufficient data to accurately plot the three f. insularia separately).

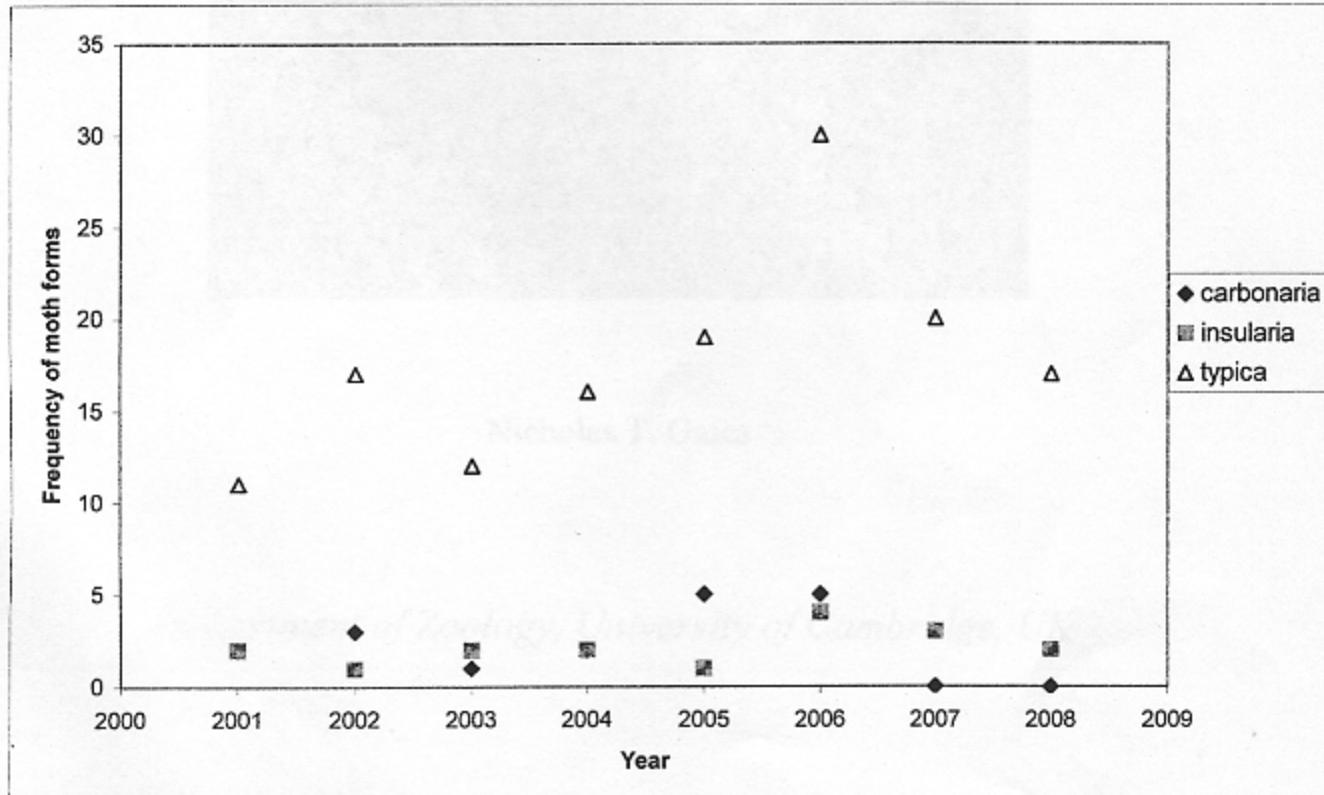


Figure 7b. Total frequency distribution per year: f. typica vs. f. carbonaria vs. f. insularia.