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Biology, Behaviour and Taxonomy of two Oleria onega subspecies (Ithomiinae, Nymphalidae, Lepidoptera) in north-eastern Peru

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THESIS ABSTRACT

Oleria onega agarista (Felder & Felder) and *Oleria onega* ssp. *nov*. are Ithomiinae subspecies that live on the Upper Huallaga River, near the town of Tarapoto in north-eastern Peru, and are separated by a mountain chain, the Cerro Escalera. *Oleria onega agarista* live on the NE slope and *O. o. spp* on the SW slope. We conducted both observational studies and field experiments to describe the ecology of these two taxa and to make hypotheses on potential selection pressures acting in the evolution of the two taxa.

1. The two *Oleria* subspecies are morphologically different and we document in the first part of this study different oviposition behaviour. Host plant preferences between four *Solanum* species, *S. mite, S. anceps. S. angustialatum* and *S. uleanum*, were tested and revealed that *Solanum mite* is the most preferred host plant by the females of both butterflies subspecies, and is also the most abundant, followed by *S. anceps, uleanum* and *angustialatum*. *Oleria onega agarista* laid their eggs on its host-plant, *Solanum mite*, whereas *Oleria onega* ssp. laid them at a distance, till up to 1 m. away from the plant. Through experiments in cages and observation in the field, it appears that this behaviour is not always the case, and that both Ithomiinae subspecies may present both behaviour, but in the field *O. o.* ssp. prefer to oviposit away from the plant, a new strategy for this genus. The main selection pressure that could lead to the oviposition behaviour differences could to be the predation on eggs and larvae, increased by competition for oviposition site between ovipositing females. The higher density of host-plant on the SW slope, allowing the larvae to find their food easier, could have helped the development of this alternative strategy.

2. Four *Solanum* species were offered to the larvae of both *Oleria* species. The results revealed that *Solanum mite* is the most preferred host by both females and immature stages. Preferences of ovipositing females and larvae are highly correlated with the performance of the larvae on *S. mite*. Egg survival in the field was tested for both *Oleria* subspecies on both side of the mountain. For this, a total of 400 eggs were glued, half of them on *S. mite* leaves and half on other substrates. No differences were found between the two butterfly subspecies, however egg survival on the SW side was higher when glued on other substrates than when glued on the host plant leaves. The *O. o.* ssp. larvae (natural of the SW side) use to move less than those of *O. o. agarista* do. Therefore a hypothesis for the behaviour of laying eggs away from the host plant was a higher predation pressure on the SW side.

Predator fauna was compared between both mountainsides, revealing that 70-80% of the potential predators were ants. Among them, *Ectatomma sp.* (Hymenoptera: Ponerinae) was found in high numbers on the SW side, but were completely absent of the NE side, where no predation events were observed on eggs. Therefore we emit two hypotheses: first *Ectatomma sp.* may eat or remove the eggs from the plant, or their presence on plants may avoid oviposition by the females.

3. Two contact zones on the SW slope are known: Ahuashiyacu, where both subspecies only cohabit, and Estero (near the village of Shapaja), where they hybridise. Genetic differences between the two subspecies and between populations were investigated with Random Amplified Polymorphic DNA (RAPD) markers. Both cluster and Principal Coordinates Analyses provided a clear but weak discrimination between the two subspecies. Genetic diversity was much higher within the populations than between them, and following the results of the cluster analysis, the geographically more distant populations were grouped. Morphological traits on the wing patterns of the hybrids are intermediary between the two butterfly subspecies, but it should be noticed that hybrids showed fewer common RAPD bands with the two subspecies than expected. Nevertheless, in the analysis they presented more similarities with *O. o. agarista*, from the Km 28 population, and only with three *O. o.* ssp. individuals from the Urahuasha populations that may be back crosses from hybrids. The individuals of the Ahuashiyacu population were well defined between *O. o.* ssp. and *O. o. agarista*, according to morphological traits, and no individual was near the hybrids, suggesting that hybridisation has not yet occurred in this population, even though both subspecies occur sympatric. As polymorphism is high (100%), the use of specific markers and analysis of other subspecies of the *Oleria onega* complex will help to determine the exact taxonomical status of *Oleria onega* ssp. and *O. o. agarista* and their hybrids. Even so, RAPD techniques provided good information for discriminating between the subspecies and among populations.

4. We attempt to model the distribution of the butterflies in relation to environmental variation, using GIS and geostatistical tools; and this part is divided in four main steps: (i) through punctual observations of twenty-three sites, cartographic maps on presence and absence of butterflies, the presence of the different host plant species, the type of soil, dimensions of trees and the density of the understorey were built. (ii) Thereafter temperature, humidity and density of the butterflies were measured for each sites. However, because of the lack of data from part of the area, synthetic data needed to be generated. Through statistical and interpolation methods, both the spatial distribution of

Oleria onega subspecies and environmental data were extrapolated to have data available for all the area. Topography and landuse were represented for all the area. (iii) Conditional simulations allowed the estimation of probabilities of presence of 1, 2, 3,4 and 5 individuals in all the study area, revealing that the probability of finding two individuals/day is closest to the actual situation. (iv) Kriging, probabilities, landuse, topography and buffers of the rivers, study sites and road were compared. The mean number of both males and females observed per day was between 0.8-1.5 individuals in most of the area. This mean number is situated mostly between 85-90% of humidity and 25-27° C. In relation to altitude, butterflies were found between 300-800m and in forested areas. In two areas, the abundance of butterflies was higher, where pathways through the mountain are possible.

RESUME

Oleria onega agarista (Felder &Felder) et *Oleria onega* ssp. *nov*. sont deux sous-espèces d'Ithomiinae qui vivent dans la région du Haut-Huallaga, près de la ville de Tarapoto, dans le nord-est du Pérou. Elles sont séparées par la chaîne de montagne appelée "Cerro Escalera" : *Oleria onega agarista* se trouve sur le versant nord-est (NE) et *O. o. spp.* sur le versant sud-ouest (SO).

1. Elles présentent des différences morphologiques et des différences dans le comportement d'oviposition. Dans la première partie de cette thèse nous avons testé leurs préférences pour quatre espèces de *Solanum,* considérées comme plantes hôtes. *Solanum mite,* qui est l'espèce la plus abondante, s'est révélée être la plante hôte préférée par les femelles des deux sous-espèces suivie de *Solanum anceps*, *S. uleanum* et *S. angustialatum. Oleria onega agarista* dépose ses oeufs sur les feuilles de la plante hôte alors que *Oleria onega* ssp. les dépose plutôt sur des objets avoisinants, jusqu'à 1 mètre de distance de la plante hôte. À l'aide d'expériences en laboratoire et d'observations sur le terrain, ce comportement s'est révélé être fréquent chez *O. o.* ssp*.,* surtout sur le terrain, bien que les deux sous-espèces d'*Oleria* en cage puissent parfois présenter les deux comportements. Le fait de déposer les oeufs loin de la plante hôte est une stratégie de ponte nouvelle, encore inconnue du genre *Oleria*. La principale pression sélective qui pourrait induire ce comportement nouveau est la présence, sur le versant SO de la montagne, de prédateurs des œufs ou des femelles entrain de pondre. Un facteur pouvant favoriser le développement de ce comportement est une densité plus grande de *Solanum mite* sur le versant SO, faciliterait ainsi la recherche de nourriture pour les jeunes larves.

2. Les quatre espèces de *Solanum* ont été présentées aux larves provenant des deux sous-espèces d'*Oleria*. Les résultats de cette expérience ont montré que, à l'instar des femelles, *Solanum mite* est aussi la plante qu'elles préfèrent. De ce fait, les préférences des femelles et de leur progéniture sont fortement corrélées entre elles, mais aussi avec les performances des larves sur *S. mite*.

La survie des oeufs sur le terrain a été testée sur les deux versants de la montagne avec les deux sous-espèces d'*Oleria*. Pour cela, nous avons placé 400 oeufs sur le terrain (200 de chaque côté de la montagne), dont la moitié ont été collés sur les feuilles de *Solanum* tandis que l'autre moitié ont été collés sur d'autres substrats, proches des plantes hôtes. La survie des oeufs collés sur d'autres substrats était bien meilleure sur le versant SO, que celle des oeufs collés sur les feuilles des plantes hôtes. Sur le versant NE, la survie était à peu près égale sur la plante et sur d'autres substrats. Aucune

différence significative a été trouvée entre la survie des œufs des deux sous-espèces. D'autre part, les larves d'*O. o. ssp* (qui proviennent du versant SO) sont plus sédentaires que celle d'*O. o. agarista*. Ces résultats laissent supposer que la pression due aux prédateurs est plus forte sur le versant SO et peut induire le comportement d'oviposition particulier d'*O. o. ssp*. Un relevé de l'épifaune prédatrice réalisé sur les deux versants de la montagne, a montré que le 70 à 80 % des prédateurs potentiels capturés étaient des fourmis. Parmi elles, un genre, *Ectatomma* (Hymenoptera: Ponerinae), a été trouvé en abondance sur le versant SO, mais était complètement absent du versant NE. Malheureusement, aucun cas de prédation sur les oeufs par ces fourmis n'a pu être démontré. Par contre nous pouvons supposer que leur présence peut affecter les papillons, soit par des actes de prédation sur les oeufs, soit en empêchant les femelles de pondre sur leur plante hôte, ce qui demande plus d'observations de terrain.

3. Bien que les deux sous-espèces de papillons soient chacune d'un côté de la montagne, deux zones de contact entre elles ont été observées sur le versant SO. Les deux sous-espèces cohabitent Ahuashiyacu , et des hybrides ont été observés a Estero. Les différences génétiques entre les deux sous-espèces et leurs hybrides, et les variations entre populations, ont été étudiées en utilisant la technique du Random Amplified Polymorphic DNA (RAPD). Les résultats, présentés sous forme d'arbre non-enraciné (UPGMA cluster), et d' Analyse des Coordonnées Principales (Pcoord), ont tous les deux démontré que les deux sous-espèces sont bien distinctes. Les hybrides, bien que morphologiquement intermédiaires, ont peu de bandes en commun avec les deux sous-espèces. Ils sont situés entre les deux sous-espèces dans l'analyse des coordonnées principales, bien que mélangés avec la population d'*O. o. agarista* du Km28 et quelques individus de celle de Urahuasha (*O. o.* ssp.*)*. Ces deux populations pourraient de ce fait présenter des signes d'hybridation passée. La diversité génétique est aussi plus grande au sein des populations que entre populations de la même sous-espèce, et des populations géographiquement éloignées sont génétiquement proches. Le polymorphisme des marqueurs génétiques neutres étant extrêmement élevé pour toutes les populations, il serait intéressant d'utiliser d'autres marqueurs spécifiques (isozymes, RFLP) et d'introduire d'autres espèces d'*Oleria* dans des prochaines analyses, ce qui nous permettrait de mieux déterminer le statut taxonomique des deux sous-espèces. Néanmoins la technique des RAPD nous a donné d'importantes informations sur la distinction entre sous-espèces, sur les similarités entre populations, et sur les variations génétiques au sein des populations.

4. Nous avons essayé de modéliser la répartition géographique des deux sous-espèces d'*Oleria* avec les données environnementales les concernant (température, humidité, topographie etc.) en utilisant les outils du Système d'Information Géographique (SIG) et de l'Analyse Géospatiale : (i) au moyen d'observations ponctuelles sur 23 sites, des cartes de distribution des papillons, de leurs plantes hôtes, du type de sol, de la taille des arbres et de la densité de végétation du sous-bois ont été créées. (ii) Les moyennes de température, humidité et densité des papillons ont été mesurées dans chaque site ; le manque de données dans certains endroits nous a poussé à injecter des données synthétiques (estimées d'après la connaissance du terrain). À l'aide de méthodes de statistiques et d'interpolation des cartes de krigeage de la densité des papillons, de la température et de l'humidité relative ont été construites sur toute la zone d'étude. Le "landuse" et la topographie ont été représentés sur toute la zone d'étude. (iii) Au moyen de simulations conditionnelles, des cartes de probabilités de présence des papillons ont été établies pour 1, 2, 3, 4 et 5 individus par jour sur toute la zone d'étude ; la probabilité de trouver deux ind. par jour s'est avéré être celle la plus proche de la réalité. (iv) les cartes de krigeage, probabilités, "landuse", topographie de même que les "buffers" construits sur les rivières, la route et les sites d'études, ont été comparés visuellement. De cette étude, nous pouvons conclure que : l'abondance des mâles et des femelles varie entre 0.8-1.5 ind./jour et est la plus répandue sur toute la zone d'étude. Cette densité se retrouve surtout entre 85-90 % d'humidité relative et 25-27° C. Les papillons se trouvent surtout dans une tranche d'altitude entre 300-800m, dans les zones forestières. Deux zones montrent une abondance plus grande de papillons. Suivant les données d'humidité, de température et de topographie, le passage des papillons est possible entre les versants, en contournant la montagne.

GENERAL INTRODUCTION

GENERALITIES ON ITHOMIINAE

The Ithomiinae are a subfamily of the Nymphalidae that include eight tribes endemic to the tropics of Central and South America : Tithoreini, Melinaeini, Mechanitini, Napeogenini, Ithomiini, Oleriini, Dircennini, Godyridini. A ninth tribe, the Tellervini, includes only one polytypic genus found in New Guinea, Celebes and Queensland (Brown, 1987b). A total of 40 genus and more than 400 species have been described. However their systematics remains unclear and very complex due to the high frequency of mimicry in the genus and to geographic variation in colour pattern. We lack molecular phylogenies for this group. Ithomiinae are medium sized butterflies, with wingspans varying between 2 and 10 cm. Traits of wing venation are the principal taxonomical characters (Lamas, in prep). The wings are either brightly coloured with brown, orange, yellow and red, or transparent with black veins and bearing some tiny hairs and no scales in the transparent parts. Males possess one or two androconial hair patches or scent scales on the dorsal costal margin of the hindwings. When males are not looking for females, their hair patches remain flat, covered by the forewing margin.

Ithomiinae prefer shady habitats and undisturbed forests. During the dry season they gather in multispecies groups with high densities of individuals (25 ind/m^2) (Brown & Benson, 1974; Brown, 1979; Vasconcellos-Neto, 1980). These gatherings are called "Ithomiinae pockets". Ithomiinae have a high dispersal ability and unlike their sister group Heliconiinae (Brown & Vasconcellos Neto, 1976; Mallet, 1986) lack a definite "home range". Their mobility and their relative longevity (up to 6 months) allow a rapid gene flow between subpopulations.

The interest in Ithomiinae began in the 19^{th} century and was due to two main reasons. First they were considered as prime distasteful models in mimicry complexes throughout the Neotropics (Bates, 1862; Müller, 1878, 1879; Brown, 1974b). More recently, because they feed on host-plants that are rich in alkaloids and other poisonous substances they represent a classical case of insect-plant biochemical coevolution (Brown et al., 1991). In the 1970's, work on biochemical particularities of Ithomiinae and Danainae were published, revealing complex insect-plant interactions (Edgar & Culvenor, 1974; Boppré, 1978; Edgar 1982) as it was discovered that pyrrolizidine alkaloids (PA's), the poisonous compounds of adult butterflies, were obtained by seeking them in flower nectar and plant exsudates, but

not from the host-plants. Primitive Ithomiinae feed on Asclepiadaceae and Apocynaceae, which contain cardiac glycosides, which are used as defences by adults. For example, *Tithorea harmonia* and *Tellervo zoilus,* which feed on Apocynaceae, are the only species that incorporate alkaloids from the larval foodplant (*Prestonia acutifolia*). The larvae of other species feed on Solanaceae which lack PA's (Brown, 1984a,b) but contain other alkaloids, steroidal bitter principles, saponins and coumarins that may be used by larvae as defences, but never by the adults (Brown, 1987a). Therefore adults need to search for other sources of PA's in other plant species. Usually males are more attracted than are females to flowers and other plant organs that promote exsudates with PA's precursors (Lamas & Pérez, 1981), and they incorporate the alkaloid pharmacophagously whereas females obtain them mostly by sperm transfer together with the nutrients used for egg production (Boggs, 1979). Some species of Asteraceae and Boraginaceae may contain PA's and are known to be attractive for adult Ithomiinae. These include *Heliotropium* sp. (Masters, 1968, Lamas & Pérez, 1981), *Eupatorium* sp. (Trigo 1990; Brown, 1984a,b, 1987a; Pliske, 1975a,b; Edgar & Culvenor, 1976), and *Epipendrum* orchids, for which Ithomiinae are the principal pollinators (DeVries and Stiles, 1990). PA's are used not only for defences but also as precursors of pheromones, that are released by the males' hair patches. The pheromones are used to attract females, but also function in territory recognition and defence, leading other males to avoid invading an already occupied territory (Pliske, 1975b). Females transfer PA's to their eggs, but larvae and pupae never contain the chemicals found in adults. Larvae are often cryptic, greenish or brownish with yellow bars, whereas pupae are metallic or with golden shiny patches (Brown & Freitas, 1994).

THE CASE OF *OLERIA ONEGA* SUBSPECIES

In this study interest was concentrated on two Ithomiinae subspecies: *Oleria onega agarista* (C. Felder and R. Felder) and *Oleria onega* ssp. The latter is a recently discovered subspecies that has not yet been described formally (Lamas, pers. com.). This new subspecies differs morphologically from *O. o. agarista* by the presence of two white bands on the forewing that are never joined (in *Oleria onega agarista* a transversal band connects this two bands on their middle part on the Cu1 and Cu2 veins) (Lamas, pers com).

Oleria onega ssp.

Oleria onega agarista

Both subspecies are endemic to the area of Tarapoto in north-eastern Peru, and are geographically separated by a mountain chain, the Cerro Escalera: *Oleria onega* ssp. lives on the SW side, whereas the NE side is the habitat of *O. o. agarista*. Two sites on the SW side were recorded as "hybrid zones" where both *O. o.* ssp. and *O. o. agarista* occur in sympatry, with cases of hybridisation. These morphological hybrids are recognised by an incomplete or absent transversal band (Lamas, pers. com.). In the lowlands of the NE side a closely related *Oleria* species, *Oleria lerida lerida,* is known and suspected to hybridize with *O. o. agarista,* but was too rare to be included in this study. Thus our study will be concentrated only on the Cerro Escalera fauna, including the two *Oleria onega* subspecies and their potential host plants.

The host plant used by the genus *Oleria* are Solanaceae (Drummond & Brown, 1987; Knapp 1997). Four Solanaceae species are suspected to be used by *Oleria onega* as host plants in the region of Tarapoto (Mallet, pers. comm.) : *Solanum mite* (Ruiz & Pav.), *S. anceps* (Ruiz & Pav.), *S. angustialatum* (Bitter) and *S. uleanum* (Bitter). Their distributions differ on the two sides of the Cerro

Escalera: on the NE slope *S. mite*, *S. anceps*, and *S. uleanum* occur in sympatry, whereas on the SW slope only *S. mite* is found. *S. angustialatum* grows on the upper part of the mountain. *Oleria* females usually lay eggs singly on the *Solanum* leaves. However, field observations suggested that *O o.* ssp. females might not oviposit on the host plant but on other objects such as stones, stems, dead leaves and other plants adjacent to the host plant. The distance between the host plant and the oviposition substrate may be up to one meter. When the female finds a host plant, two different reactions are possible: the first is to quickly lay an egg under the leaf (mostly young leaves but not always), and the second is to keep searching for a place on another substrate near the host plant. The behaviour of not laying eggs on the host plant is mostly related to abiotic environmental factors in temperate climates (Wiklund, 1984) and has not been previously reported in Ithomiinae. We suspected that this behaviour may be related to differences in abundance of natural enemies. Therefore the oviposition behaviour of both *Oleria* subspecies was studied and compared.

In the field of butterfly-host plant relationships, the most frequently studied subjects are host specificity and host plant switches (Wiklund, 1975; Williams, 1983; Futuyma & Moreno, 1988; Thompson, 1996), while specific oviposition behaviour in relation to external factors (climate predators etc..) is less well studied. The few studies performed on this topic are mostly related to climate and host plant abundance (Wiklund, 1984; Higashiura, 1989; Bergman, 1996; Steiner & Trusch, 2000). The relation between adult oviposition preference, and on one hand larval host choice, as well as larval success on the other, are closely related and both are taken into account in studies of plant-insect relationship (Thompson, 1988; Nylin, 1993; Pires et al., 2000; Craig et al., 2000, Harris et al., 2000). Here we will concentrate our interest on larval performance and behaviour, and the relation of these parameters to female oviposition behaviour.

THESIS OUTLINE

The major aim of this study was to investigate the particular oviposition behaviour of *Oleria* females. As we were not sure that the four *Solanum* species known from the literature are used as host plant by the two butterfly subspecies, we tested the host plant preferences of both females and larvae, as well as larval performance on the different *Solanum* species. Development of immature stage will be described, as well as eggs, larval behaviour and larval survival, which are closely related to female oviposition choice. However, it was also necessary to clarify the genetic relationship between the two subspecies we studied and their morphological hybrids.

The four chapters treated in this thesis are closely linked. The first step is the study of comparison of oviposition behaviour and host plant preferences for four *Solanum* species of the two subspecies. These results led to further questions concerning larval behaviour, preferences, performances and their relation with other environmental factors such as host plant density, diversity, and predator fauna. Thereafter, the genetic relationships between the two subspecies were studied to assert that we have two genetically different groups. As gene flow exist between the different populations studied, we tried to understand the relationship between butterfly distribution and abundance and their environmental factors, and to indicate sites where both subspecies may meet. As a function of temperature and climate. GIS tools were used for cartographic representation of the butterflies and host plant distribution, and Geospatial Analysis tools for the modelling of the different environmental factors and butterfly density, allowing the study of their relations.

The present thesis focuses on the following questions:

1. *Are the four Solanum species host plants of Oleria onega agarista and O. o.* ssp*? How does oviposition behaviour of the two Oleria subspecies differ? Solanum mite, Solanum anceps, Solanum angustialatum* and *Solanum uleanum* are found in the area of the Cerro Escalera and are supposed to be host plants, as the genus *Solanum* is usually associated with the genus *Oleria* (Drummond & Brown, 1987; Knapp, 1997). In this study the aim was to evaluate the preferences of the females for the four host-plants and thereafter to study the preferences between oviposition sites (on leaves of the host plant or on other substrates) for both butterfly subspecies. We also wanted to understand the

factors inducing the behaviour of laying eggs close to the host plant through field observations and laboratory experiments.

2. *Is oviposition preference correlated with preference and performance of the larvae? Is there a difference in potential predation pressure on larvae of both subspecies, that may affect their behaviour? Is there differences in potential predators species for different oviposition environments?* Preference and performance of the larvae were tested on the four host plant species and correlated with the results presented in Chapter 1. Survival of eggs in the field that were laid on the host plant leaves was compared with survival of eggs laid on other substrates for both sides of the mountain. This was done to investigate the potential relationship between egg survival and the laying of eggs close to rather than on the host plant leaves. Larval behaviour and circadian activities were also compared between both subspecies. The potential predator groups were identified and compared between both sides of the mountain.
3. *Are the two Oleria subspecies genetically distinct?* The first aim was to define if *Oleria onega* ssp. and *Oleria onega agarista* are genetically distinct, but also if differences occur between the different populations studied, and if gene flow occurs. “Morphological hybrids” between *O. o.* ssp and *O. o. agarista* were observed in the field, but we could not be sure that they were truly intermediate forms, rather than a new differentiation of one subspecies. The different populations are geographically separated by mountains or by deforested areas, but we did not know if the geographically close populations were also genetically close. We selected RAPD techniques for a preliminary molecular approach because they provide a virtually unlimited number of anonymous DNA markers (Williams et al, 1990) and are therefore appropriate for initial, overall analysis of variation between populations.
4. *Is there a relationships between distribution of Oleria and environmental factors such as altitude, temperature, humidity and other factors?* This aspect was investigated by GIS (Geographical Information System) and Geospatial Analysis tools, allowing us to model the relationship between butterfly abundance, temperature, humidity and other environmental factors. Even though the Cerro Escalera range constitutes a biological barrier, individuals of *O. o. agarista* were found on the SW side. Therefore we suppose that butterflies of the NE side had found a pathway around the mountain, that depends on the topography, environmental and climatical factors. Through cartography of the different factors, we wanted to find a relationship between organisms and their environment that may induce the contact between subspecies. This part is divided in four main steps: (i) to build maps on

presence and absence of butterflies, the presence of the different host plant species, the soil type, the tree size and the density of the understorey; (ii) thereafter, the mean temperature, humidity and density of butterflies was measured for each site and was calculated by kriging to extend the data to the whole study area (iii) conditional simulations allowed us to build probabilities maps (iv) results of kriging, probabilities, landuse, topography and butterfly data were compared.

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Chapter 1

Host-plant preference and oviposition behaviour of two Oleria onega subspecies (Ithomiinae, Lepidoptera) in north-eastern Peru.

Based on:

Gallusser, S.; Rahier, M.

Host-plant preference and oviposition behaviour of two *Oleria onega* subspecies (Ithomiinae, Lepidoptera) in north-eastern Peru. In preparation for submission to *Biological Journal of Linnean Society*

INTRODUCTION

Butterflies are among the most studied groups of insect in relation to their oviposition behaviour and host-plant selection. Gilbert and Singer (1975) reviewed the principal traits of their ecology, Chew and Robbins (1984) their egg laying behaviour, Thompson and Pellmyr (1991) the evolution of their oviposition behaviour, Jaenike (1990) used them as model organism to study host specialisation and genetic and non-genetic causes of variation in host specialisation. Despite of the large number of publications on these subjects, new behavioural traits are still being found (Karlsson, 1995).

Several factors can act to shape selection on oviposition behaviour. Among these, larval performance and survival, availability of adult resources (nectar or mating sites), distribution of predators with respect to potential host plants, plant morphological characteristics, and density and dispersion of host plants have all been discussed in the literature (Rausher, 1978, 1979; Tiritilli & Thompson, 1988; Nylin & Janz, 1993) revealing that larval success is not always the main pressure for female decision. To recognise a plant suitable for its offspring, the female uses a variety of cues. The plant recognition process followed by the gravid female is similar in most butterfly species, and can be divided in two main components (Hern et al., 1996; Renwick & Chew, 1994):

a) Pre-alighting behaviour : consists of searching, orientation and encounter: Females are attracted to their host plant by chemical cues (Stadler et. al, 1995; Oyeyele, 1990; Pereyra & Bowers, 1988; Honda, 1990) and / or visual cues such as leaf shape and colour (Gilbert, 1975; Rausher, 1978; Forsberg, 1987). Generally the balance between positive and negative chemical signals is important, and therefore acceptance is not typically dictated by only one substance. The colour of the plant also reveals its physiological condition. Butterflies which use visually apparent host plants usually seem to find them without alighting on non-hosts (Wiklund, 1984).

b) Post-alighting discrimination characterised by landing, plant evaluation and acceptance or rejection. Landing on a plant by a gravid female is the transition between pre-and post-alighting behaviour. The contact evaluation on the plants' surfaces proceeds very rapidly, and involves sensory receptors located on of the forelegs, proboscis, antennae and ovipositor (Renwick and Chew, 1994). In some butterflies such as Nymphalids, the forelegs are reduced to small appendages used only as tactile organs and chemosensory (Calvert, 1974).

Once a host plant is accepted, females have to find the best place on (or near) the plant where they can lay their eggs; nevertheless they are often found to oviposit on sites not optimal for the fitness of their offspring (Rausher, 1979; Chew & Robbins, 1984; Higashiura, 1989). In fact some butterflies are known not to oviposit on their host plants: for example, some grass-feedings satyrids drop their eggs on the ground with or without previous plant recognition (Bergman, 1996; Steiner & Trusch, 2000) whereas females of *Parnassius apollo* lay eggs at 1 to 2 m away from the host plant, sometimes without previous recognition of the host plant (Wiklund, 1984). This is a common pattern in temperate areas for butterfly species that spend the winter in the egg stage and that have herbs as host plants. Up to now, the behaviour of laying eggs away from the host plant has not been reported for tropical butterflies.

We examined in this study the oviposition behaviour of two Ithomiinea butterfly subspecies. Some early studies on Ithomiinae concern their ecology (Haber, 1978; Drummond, 1976; Drummond & Brown, 1987; Masters, 1968). Recently, interest was focused on their mimetic qualities (DeVries et al., 1997, Devries & Lande, 1999; Beccaloni 1997), biology (Brown, 1994), and their chemical particularities (Brown, 1976 1984a,b, Brown & Vasconcellos-Neto1987, Cardoso, 1997, DeVries et al., 1997, Pinheiro 1996), but oviposition behaviour of these butterflies has been neglected. Oviposition behaviour differs strictly between *Oleria onega agarista* (Felber & Felber) and *Oleria onega* ssp. nov. (a recently discovered subspecies). Females of *Oleria onega* ssp. have been observed to recognise the plants by alighting on them, but they search in the neighbourhood for other places to deposit their eggs (Mallet, pers. comm.), probably taking into account a variety of unknown parameters, whereas the scarce observations done on *O. o. agarista* suggest that females oviposit directly on the leaves of the host plant.

Oleria onega is a complex of some fifteen subspecies (Lamas, pers. comm.) found throughout the South-American tropical forests. Some subspecies have not yet been described in detail. Two of these subspecies, investigated in this study, are present in the area of Tarapoto (S 06°22'50'; W 076°26'23''), in northeastern Peru: *O. o. agarista* and *O .o.* ssp*.,* a recently discovered subspecies whose status is still not clearly defined (Lamas, pers. comm.). Two other subspecies are present near the area, but are too rare to be taken into account in this study. In some localities (Ahuashiyacu and Shapaja, see below for site information) (Fig. 1) they are sympatric, and may hybridise (Lamas, pers. comm.) (Chapter 3). However, these two subspecies are mostly geographically separated by a mountain chain, the “Cerro Ecalera”, which constitutes a strong biological barrier between areas with

considerably different faunas and floras (Joron 2000; Joron et. al. 2001, Shulte 1999, Mallet, 1989, 1993); *O. o.* ssp.. is mostly found on the south-western slope, whereas *O. o. agarista* extends its range on the north-eastern slope (thereafter termed as SW and NE slopes) and all the Huallaga Valley. Four species of Solanaceae are expected to be used by *Oleria onega* as host plants (Drummond & Brown, 1987; Knapp & Helgason, 1997) : *Solanum mite* (Ruiz & Pav.), *S. anceps* (Ruiz & Pav), *S. angustialatum* (Bitter), and *S. uleanum* (Bitter) (Knapp & Helgason, 1997). On the NE slope, three of them occur in sympatry: *S. mite*, *S. anceps*, and *S. uleanum*, whereas on the SW slope only *S. mite* is found. *S. angustialatum* grows on the upper part of the mountain. In the *O. o.* ssp. habitat (SW slope), where only *Solanum mite* is found, the abundance of host-plant (in the sense of both density and area covered) is also higher than on the other side of the mountain. Plants of *S. mite* grow in secondary vegetation, mostly on the borders of the paths, and as deforestation is greater on the SW slope, open areas are wider and allow bigger plant patches.

The behaviour of laying eggs on places other than the host plant had not been previously observed in Ithomiinae. The evolution causes and the costs and benefits for the females and their offspring, are unknown.

In this study the first aim was to examine host-plant preferences and host location behaviour of each subspecies. We also evaluate how density and diversity of the *Solanum* affect the oviposition behaviour, oviposition choice and the proportion of eggs laid on or near the plant. This is relevant here because habitat occupied by the two subspecies exhibit a marked difference in diversity of potential host plants. We document that *O. o.* ssp. lay eggs mostly on substrate other than plants and that the preferred host plant for both subspecies may be *Solanum mite* as being the commonest and most abundant one. Our second aim was to describe and quantify the differences in oviposition behaviour between these two butterfly subspecies. Hypotheses to explain oviposition away from the host plant will be proposed.

MATERIALS AND METHODS

Study organisms: *Oleria onega agarista* (Felder and Felder, 1862) and *Oleria onega* ssp*.* are considered as conspecifics (Lamas, pers. comm.). *O.o. ssp..* differs morphologically from *O. o. agarista* by a narrower black edge of the wings, and by the presence of two white bands on the forewing that are never joined (Lamas, pers com). Whereas in *Oleria onega agarista* a transversal band connects these two bands on their middle part on the Cu1 and Cu2 veins. Morphological intermediates were recognised by an incomplete or absent transversal band. Eggs are laid singly after plant recognition and this stage lasts on average three days. Five larval instars are followed by pupation. Larval development requires approximately seventeen days and pupation seven to eight days. Oleria may reached some six to eight generation per year.

Study sites (fig. 1): The study sites are situated between Tarapoto (SW slope) and Km 30 of the road (Carretera marginal) to Yurimaguas (NE slope) (S 06°24- 06°28 and W 76°18 – 76°22). The road crosses the Cerro Escalera mountain by a tunnel (alt. 1100 m), and there is a great contrast in climate between the two sides of the mountain. The SW side is usually sunny and hot (mean temp. ca. 30°C), while in the other side, the climate is wet and rainy, including fog and cooler temperatures (mean temp. ca. 27° C).

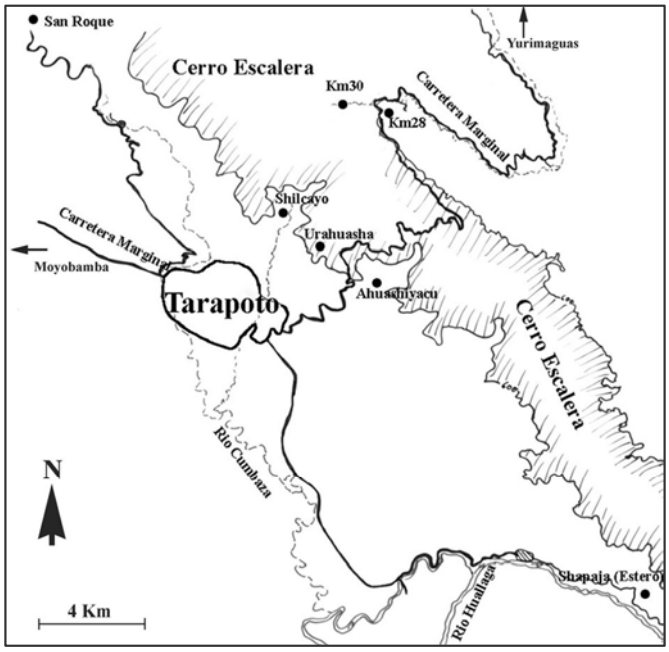

Figure 1 : Geographical distribution of the field sites *of Oleria onega ssp.,* and *Oleria onega agarista* in North-eastern Peru.

Females of *Oleria onega ssp.*. were collected and followed in three sites on the SW slope, Shilcayo, Urahuasha, Ahuashiyacu and *Oleria onega agarista* was studied on two different sites on the NE side, Km28 and Km30. In Ahuashiyacu, both subspecies meet, but very few *O. o. agarista* individuals were observed. Because of their slow flight and the fact that their are found in low vegetation, females are relatively easy to follow in the field, but even so vegetation sometimes hinders long pursuits. In our field observation, each step of the recognition and oviposition behaviour will be detailed : pre-alighting recognition, plant evaluation by tasting and egg laying.

Observation of females in the field

Preliminary observations on Ithomiinae (unpublished results) revealed that the best time for field observations was in the morning and at the beginning of the afternoon, between 9.00h and 13.00h. A total of ninety-one gravid females were followed during three field seasons (between Oct. 1998 and Jan. 2001), in five sites : Ahuashiyacu, Shilcayo, Urahuasha, Km 30 and Km28. The following information was noted for each female: distance that the female flew between the recognised host plant and the oviposition site, the minimum distance that the larva had to move to reach the host plant, the substrate on which eggs were laid and the time laps taken by the female between host plant recognition and oviposition. What we term the “distance of the larvae”, is the minimum distance that the larvae have to wander between the support where it hatched and the nearest host plant. This distance was estimated following the shortest way to reach the plant, supposing that the larvae walk on the ground, on sticks or other objects between the support and their first food. The “distance of the female” is the shortest flying distance between the host plant and the support where the female laid the egg. The distance of the female and the distance of the larvae were compared for three *O. o.* ssp. populations and analysed by a Kruskal Wallis test. The differences between the substrates chosen by both butterflies subspecies were analysed with Fisher's exact test. The mean time taken by both *Oleria* subspecies between plant recognition and oviposition were compared by a *t*-test (Sokal & Rolf, 1995).

Oviposition experiments with females in cages

Experiments in flight cages, conducted in Tarapoto, were realised to allow an easier manipulation of the host plants and the butterflies, and to provide a more controlled environment to

define the host preferences and the oviposition sites preferences of the females. Butterflies were kept in cages constructed with wood and wire-mesh (plastic and metallic) stapled to the wood. To reduce mortality due to ants, the cages were standing raised above the ground. Cage size was $1m^3$, which allowed the butterflies to fly freely. Constant access to small recipients with 10% sucrose solution was insured in the cage. *Heliotropium* flowers were regularly put into the cage as a source of alkaloids (PA's), as well as bird dropping as an amino acid source for egg production.

The *O. o.* ssp. females used in cage experiments originated from the Urahuasha, Shilcayo and Ahuashiyacu populations (on the SW slope), and the *O. o. agarista* females from the Km30 population (on the NE slope).

Screening experiments

The screening experiments were conducted in order to study the place on or away from the host plant where eggs were laid, and to define clearly whether the two *Oleria* subspecies have different egg-laying behaviours.

Five to fifteen individuals of each host plant species were collected and potted to facilitate their manipulation. Females of *O. o.* ssp. (from Shilcayo) and *O. o. agarista* (from Km30) were separated in two cages, with 10-15 females per cage. Within each cage, one individual of each of the four plant species (*Solanum mite*, *S. anceps*, *S. angustialatum* and *S. uleanum*) was placed in each corner. Every two days, eggs were collected and counted, and we recorded the number of eggs laid on each host plant, or on objects in their vicinity (i.e., within a distance of 40 cm of the plant) (Schöps & Hanski, 2001). The radius circle of 40 cm, was chosen as at least 80 % of the eggs were observed to be laid within this distance of the plant in the field.

Female preference:

The relative preferences of each butterfly subspecies for the two most common host plants, *S. mite* and *S. anceps*, was tested with a single female per cage. In each cage, we placed two host plants (one of each species) and two non-host plants, randomly selected in the habitat used as controls. The plants were placed in each corner of the cage. Their positions were randomly distributed but an alternation between host and non-host plant was maintained. The plants used as controls aid in determining if a female recognises a host plant and oviposits on others or not. Every day, plants were

changed in the cage, so that each day females were offered a new treatment. Females were also changed daily. However because not enough females were available, some of them were used two or three times. During 14 days, eggs were collected each morning, and the position of each egg on or near the plant was registered. Eggs encountered on other substrates within 40 cm from a plant, were considered as associated with this plant. Data were analysed for each butterfly subspecies, applying Kruskal Wallis test (Sokal & Rohlf, 1995) to test for differences in proportions of eggs layed on *S. mite* and *S. anceps*. Differences between the number of eggs laid on leaves and on other objects was analysed with Mann-Whitney test (Sokal & Rohlf, 1995) for each plant.

Effect of host plant density and diversity on female oviposition choice

As host plant diversity and density differed between the NE and SW sides of the Cerro Escalera, two series of experiments were conducted in order to determine whether host plant density and species diversity affect on female oviposition choice and the number of eggs laid per female. Host-plant biomass density was measured for habitat on the two sides of the mountain (unpublished data). On the South-western side, only *Solanum mite* was found, growing in big patches (of 20 to 100 plants), and showing a higher leaf density per square meter than in the patches on the NE slope, where a higher species diversity was found with *Solanum mite*, *S. anceps* and *S. uleanum*. The SW slope was therefore characterised by a high density and low diversity, whereas the NE slope was characterised by a low density and high diversity. Patches were also smaller (1 to 10 plants) on the NE slope. For our experiments we used several treatments with different numbers of plants and different proportions of the various species. For all the experiments described here females from the Shilcayo (SW) and Km30 (NE) populations were used, being changed every two days. However, because few females were available for the experiment, some of them were re-tested using different treatment. Plants were also changed each two days. Eggs were collected, and we recorded whether they were laid on the host plant (and on which plant species) or on objects near (up to 40 cm) the host plant. Eggs encountered on other substrates within 40 cm from a plant, were considered as belonging to this plant, allowing a neutral zone of at least 20 cm (cage sides were 1m long) between the different plants (i.e. plant plus a 40 cm radius circle). No control (non-host plant) was used in these two experiments. Females which did not lay eggs during the experiment were discarded.

Four treatments were designed using two densities of two plant species (*S. mite* and *S. anceps*), with one treatment per cage. The treatments had the following plant composition: 4 *Solanum mite*, 1 *S. mite*, 4 *S. anceps*, 1 *S. anceps*. For the treatments with four plants, one plant was placed in each corner of the cage, and for the treatments with a single plant, the plant was placed on the centre of the cage. Thus, four cages were used for each *Oleria* subspecies. The experiment was replicated ten times with different females. Eighteen *O. o.* ssp. and nineteen *O. o. agarista* females were used. The proportion of eggs on the leaves divided by the total number of eggs laid in each cage were calculated for each treatment and analysed by a Kruskal Wallis and by a Mann-Whitney test.

Five "diversity" treatments were designed with the following proportions of plants: A = 4 *Solanum mite*, B = 3 *S. mite* + 1 *S. anceps*, C = 2 *S. mite* + 2 *S. anceps*, D = 1 *S. mite* + 3 *S. anceps*, E = 4 *S. anceps*. For all these treatments one plant was placed in each corner of the cage. Five cages were used for each *Oleria* subspecies. This experiment was replicated thirteen times with one treatment in one cage. Fifteen *O. o.* ssp. and nineteen *O. o. agarista* females were used. For each of the treatments and subspecies, the following quantities and proportions were calculated :

a : The total number of eggs laid on each treatment by each female. Differences between number eggs laid on the five treatments were analysed by a Kruskal Wallis test for both butterflies subspecies. For each treatment, the differences between the two butterflies subspecies were analysed by Mann-Whitney tests.

b : Proportion of eggs laid on leaves, regardless of the plants species, out of the total number of eggs laid for each treatment. Differences between the five treatments were analysed by a Kruskal Wallis test for each butterflies' subspecies. Differences between the subspecies were analysed by a Mann-Whitney test for each treatment.

c : Proportion of eggs laid on leaves of *S. mite* and *S. anceps*, out of the total number of eggs laid for each treatment. For each of the treatments B, C and D, that included both plant species, a Mann-Whitney test was performed to examine the difference between plants.

RESULTS

Observations of females in the field

Females ready to lay eggs are easily recognisable because of their slow flight and their plant searching behaviour. They appear to recognise the host plant species first visually, then land and examine the leaves quickly with their forelegs. However, females do not always accept the host plant by the visual or chemical signals. Sometimes they touch all the leaves (of a host and a non-host plant) that they find on their way and continue to fly until they find a suitable plant, suggesting the importance of contact recognition. An estimation of 80 % of plant individual accepted was true for all the studied *Solanum* species. Once they locate a suitable host plant individual (in a patch of ten plants, sometimes only one is chosen by several females), they show one of two different responses: either they quickly lay the egg on the under side of the leaf (mostly but not always young leaves), or they continue to look for an ideal place near the plant. Sometimes they appear to be disoriented and have to go back to the plant to taste it again before they find an acceptable substrate for their egg. The substrate can be dead material near the ground, other plants (non-host), stems or rocks, but never higher than the host plant (max 1.5 m). Eggs were never observed to be laid more than one meter away from the plant but even at this distance, reaching their first meal might require a dangerous and long trip for the newly hatched 1 mm caterpillar .

The *Oleria onega* ssp. females, in Ahuashiyacu, Shilcayo and Urahuasha (79 obs.), laid their eggs almost always near the host-plant, on stones, stems, dead leaves, sometimes even 1m away form the host plant. In contrast, *Oleria onega agarista* females (12 obs) , in Km30 and Km 28, laid their eggs on the undersides of the *Solanum mite* leaves. The mean time lapse between plant recognition and oviposition was 2.7 minutes (stdv. = 0.17) for *O. o.* ssp. and 1.7 minutes for *O. o. agarista* (stdv. = 0.25), but these two means were not significantly different ($T = 1.78$, $p = 0.08$). Figure 2 compares two distances between oviposition site and host plant, for three different populations of *O. o.* ssp.. The distances that the females flew showed more variation than the distance that the larvae had to wander. There were no significant differences among the three populations (Kruskal-Wallis : $p > 0.1$ for the distance of the larvae, and $p > 0.3$ for the distance of the female). The standard deviations were very large, revealing great variation among individuals, and these probably led to the insignificant differences among populations even if distances of Ahuashiyacu populations appear longer than those of the others

two. The preferred oviposition site for *O. o. agarista* was on the host plant (Fisher's exact test: p = 0.001) (fig. 3), nevertheless some eggs were laid on dead material. For *O. o.* ssp. the preferred sites were dead material on the ground and some eggs were also laid on non-host plants (species randomly chosen by the females). For both butterfly subspecies, laying eggs on other substrates always followed a previous alighting on the nearest host plant.

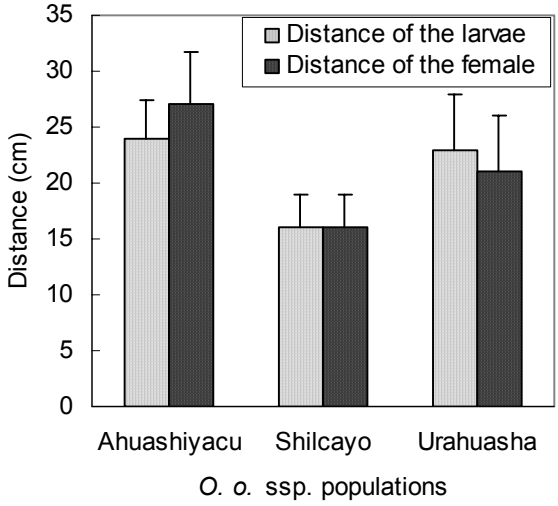

Figure 2 : results from the observations on oviposition in the field for *O. o.* ssp. (n =79 obs.).
Bars indicate the mean distance travelled by the female between the recognised host plant and the support where the egg is laid, and the estimated mean distance for the larvae to wander for three *O. o.* ssp. populations : Ahuashiyacu, Shilcayo and Urahuasha. Differences between the mean distances of the larvae and the female were not significant between populations (Kruskal - Wallis : $p > 0.2$ for both distances).

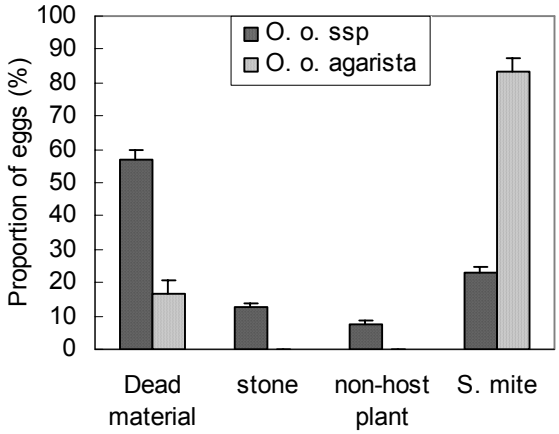

Fig 3 : Results from the oviposition observations in the field for *O. o.* ssp. (total number of eggs laid = 79) and *O. o. agarista* (total number of eggs laid = 12 obs.).

Bars indicate the mean proportion of eggs laid by the females of each subspecies on the three main supports (dead plant material, stone, non-host plant) and on the host plant, *Solanum mite*. Differences between the preferences of *Oleria* subspecies for different substrates were highly significant (Fisher's exact test : p = 0.001).

Oviposition experiments with females in cages

Screening experiments

Solanum mite was the most preferred host plant for both subspecies (fig 4 A and B). *O. o. agarista* oviposited mostly on leaves or at least an approximately equal proportion of eggs on leaves and other substrates, while *Oleria onega* ssp. laid more eggs on other substrates near the host plant. The same pattern was fared for all plants except for *S. uleanum* which was used only by *O. onega* ssp., and eggs were laid predominantly on the leaves of the plant (fig. 4A). Because of the variable number of females per cage, these results are not quantitative and no statistical tests were performed on them.

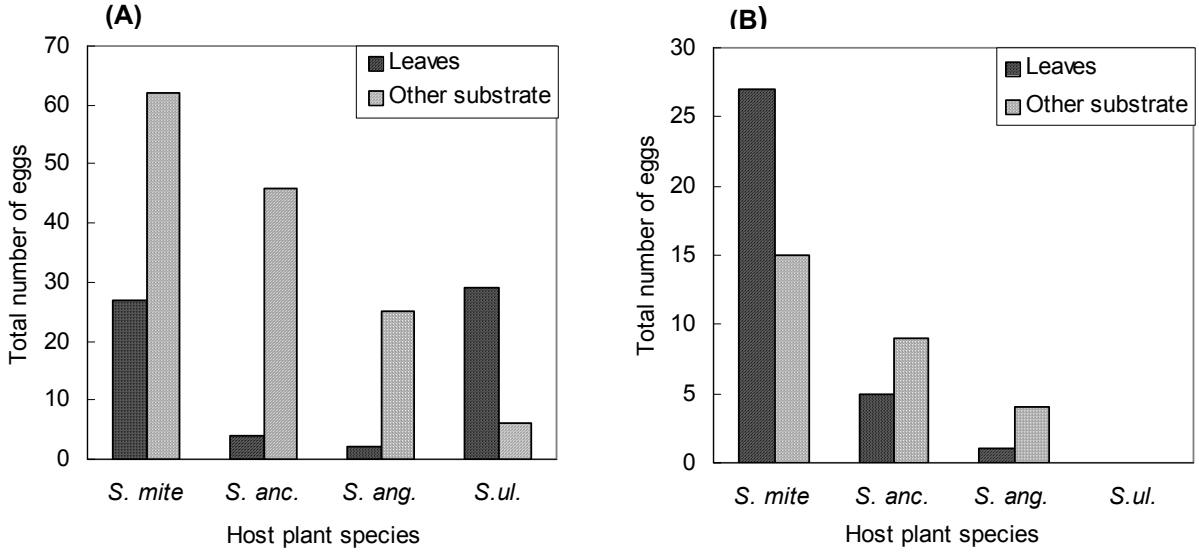

Fig. 4 A et B: Results from a choice test conducted with several females (10 - 15) per cage with four host plant species (*Solanum mite, S. anceps, S. angustialatum, S. uleanum*) for (A) *Oleria onega ssp.*, and (B) *Oleria onega agarista*. No statistical tests were performed on these data due to the variable number of females per cage. Eggs were laid on host plants or on other objects such as cage walls and the plant's support.

Female preference:

Both *Oleria* subspecies laid significantly more eggs on *S. mite* than on *S. anceps* (Kruskal-Wallis test for *O. o.* ssp. $H = 8.49$ d.f. = 2; $P = 0.014$, and for *O. o. agarista* $H = 8.03$ d.f. = 2; $P = 0.018$). No eggs were laid on the control plant (Fig. 5A and B).

Results of Mann-Whitney tests performed on the proportions of eggs laid on the host plant and on objects near the host-plant (within a distance of 40 cm) by each butterfly subspecies, revealed a significantly greater proportion of eggs laid on *S. mite* leaves by *O. o. agarista* ($U = 4.5$, p = 0.03). *O. o.* ssp. laid eggs in similar proportion on the leaves and on other substrates for both host plants (*O. o.* ssp. : *S. mite*: $U = 3.5$, p= 0.19, and *S. anceps* $U = 6$, p = 0.56). For *O. o. agarista* as well, no difference was found between the proportion of eggs laid on the *S. anceps* leaves and on other substrates nearby ($U = 12$, p = 0.33).

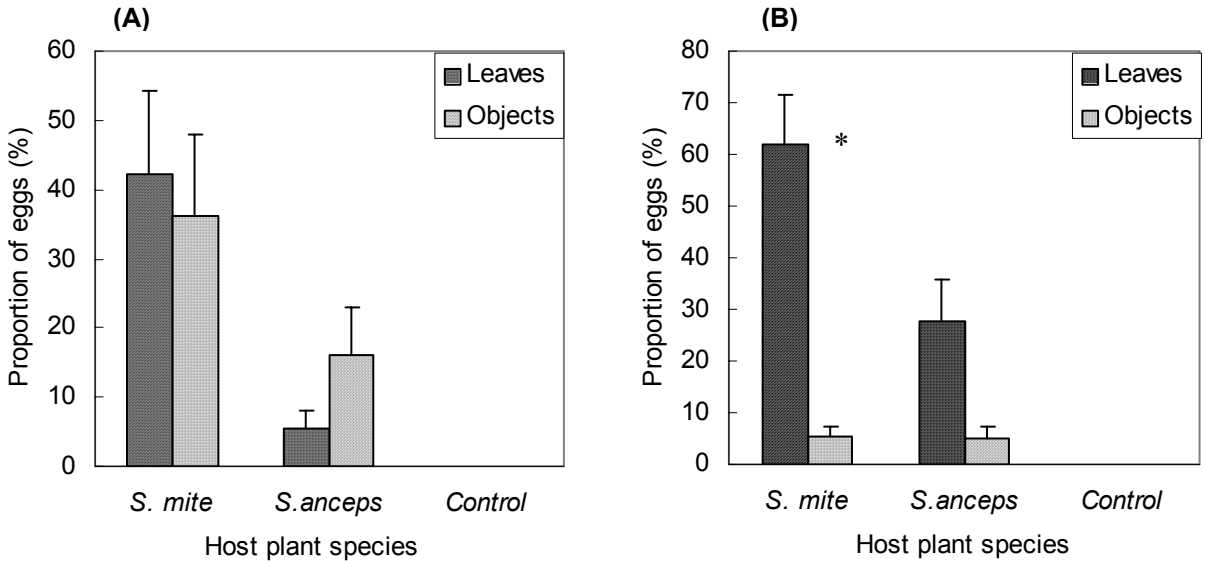

Figure 5 : Results from a choice test with single females of (**A**) *O. o.* ssp. and (**B**) *O. o. agarista* per cage (n = 24 obs. for *O. o.* ssp. and n = 36 obs. for *O. o. agarista*).
Females were offered two host plant species and two non-host plants as control. Eggs were laid on leaves and other objects (bags and cage walls). Differences between the proportions of eggs laid on *S. mite* leaves, objects near *S. mite*, on *S. anceps* leaves, and objects near *S. anceps* were significant for both *Oleria* subspecies (Kruskal Wallis : **Fig.5A:** $H = 8.49$ d.f. = 2; $P = 0.014$ p.; **Fig 5B** : $H = 8.03$ d.f. = 2; $P = 0.018$). Differences between the different proportions of eggs laid on leaves and objects for each host plant were only significant for *O. o. agarista* on *S. mite* (* indicates the significant differences) (Mann - Whitney: **Fig. 5A** : *S. mite* : $U = 3.5$, p= 0.19, and *S. anceps* : $U = 6$, p = 0.56, **Fig 5B** : *S. mite* :$U = 4.5$, * p = 0.03, and *S. anceps* : $U = 12$, p = 0.33)

Effect of host plant density and diversity

Non-host plants were not necessary as controls in this experiment, as no eggs were found on them in the previous experiments.

a) "Density" : four treatments tested : A = 4 *Solanum mite*, B = 1 *S. mite*, C = 4 *S. anceps*, D = 1 *S. anceps.*

For both *Oleria* subspecies no significant differences were found in the proportion of eggs laid on the leaves in different density treatments, regardless of host plant species (Kruskal Wallis test p > 0.2 for *Oleria onega* ssp. and *Oleria onega agarista*). For both plant species and both *Oleria* subspecies differences between the proportion of eggs laid on leaves under the two host plant density were not significant (Mann-Whitney test : *O. o.* ssp : between treatment A and B: p = 0.67, between treatment C and D : p = 0. 96; *O. o. agarista* : between treatment A and B: p = 0.42, between treatment C and D : p

= 0. 35). For some treatments, not enough data could be collected, because females laid an insufficient number of eggs, and replicates were not in similar proportions. Because of the non-significant results, no further analyses were performed on these data.

b) "Diversity" : Five treatments tested : A = 4 Solanum mite, B = 3 S. mite + 1 S. anceps, C = 2 S. mite + 2 S. anceps, D = 1 S. mite + 3 S. anceps, E = 4 S. anceps.

For O. o. ssp. the total number of eggs laid on each treatment differed significantly with a maximum on treatment C and a minimum on treatments A and E (Kruskal-Wallis test : H = 9.97 d.f. = 4; *P* = 0.041) No significant differences were found for *O. o. agarista* (Kruskal-Wallis test : H = 6.84 d.f. = 4; *P* = 0.14) (Fig 6). The mean number of eggs laid in each treatment varied between 2.5 and 4.5 for *O. o.* ssp.*,* and between 2,2 and 3.8 for *O. o. agarista*. No significant differences were found between the total number of eggs laid by the two butterfly subspecies for any of the treatments (Mann-Whitney test: p > 0.1 for each treatment).

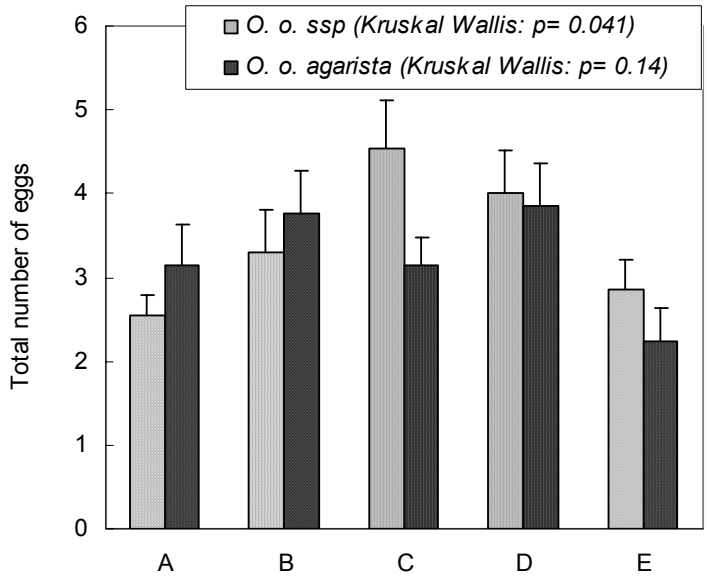

Figure 6 : Results from a choice test with *O. o.* ssp. (15 females, n = 65 obs.) and *O. o. agarista* (19 females, n = 65 obs) with different proportions of the two host plants in five different treatments.
Five diversity treatments were designed, with one treatment in one cage with the following proportions of plants: A = 4 *Solanum mite*, B = 3 *S. mite* + 1 *S. anceps*, C = 2 *S. mite* + 2 *S. anceps*, D = 1 *S. mite* + 3 *S. anceps*, E = 4 *S. anceps*. The total number of eggs laid on leaves and other substrates was compared between the five treatments for both butterflies subspecies with significant results only for *O. o.* ssp. (Kruskal Wallis : *O. o. ssp.* : H = 9.97 d.f. = 4; *P* = 0.041 and *O. o. agarista* : H = 6.84 d.f. = 4; *P* = 0.14). Differences between both subspecies were analysed for each pair of treatment with no significant results (Mann-Whitney : P > 0.1 for the five treatments).

The different plant proportions did not seem to affect considerably the proportions of eggs laid on the leaves of the host plant on the different treatment (Fig 7). For both butterfly subspecies 60 - 85 % of the eggs were found on the leaves. The results of the Kruskal Wallis test performed between the proportions of eggs laid on leaves in the five treatments were non-significant for both *Oleria* subspecies (*O. o.* ssp.: H = 4.09 d.f. = 4; *P* = 0.39 and *O. o. agarista* : H = 1.6 d.f. 4; *P* = 0.8) (Fig 7). The results of the Mann-Whitney tests performed between the proportion of eggs laid on leaves in each treatments by both *Oleria* subspecies were only significant for treatment D (*U* = 33, p < 0.01) (Fig 7) where *O. o.* ssp. laid fewer eggs on the host plant leaves than on other substrates.

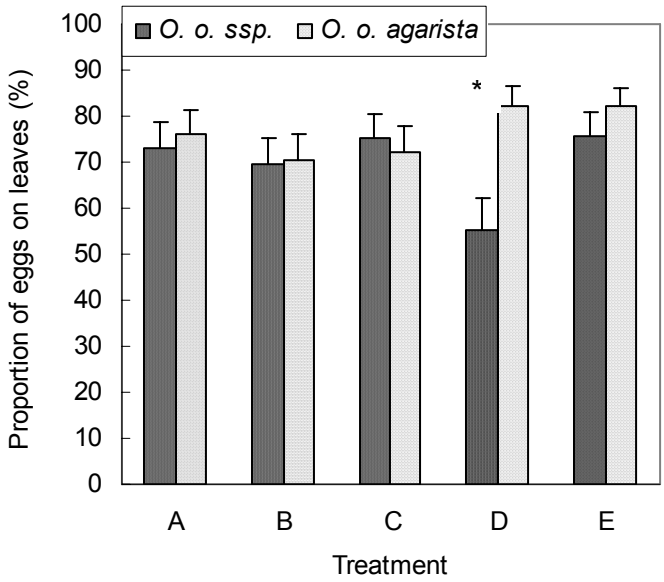

Figure 7 : Results from a choice with *O. o.* ssp. (15 females, n = 65 obs.) and *O. o. agarista* (19 females, n = 65 obs) test with different proportions of the two host plants in five different treatments
Five diversity treatments were designed, with one treatment in one cage with the following plant proportions: A = 4 *Solanum mite*, B = 3 *S. mite* + 1 *S. anceps*, C = 2 *S. mite* + 2 *S. anceps*, D = 1 *S. mite* + 3 *S. anceps*, E = 4 *S. anceps*.

The proportions of eggs on the leaves of both host plant were compared between each treatments for both butterflies subspecies with no significant results (Kruskal-Wallis p > 0.4 for both *Oleria* subspecies). Differences between both subspecies were analysed for each treatment with significant result only for treatment D (Mann-Whitney : treatment D: *U* = 33, * p < 0.01, others treatments p > 0.1).

The differences between the proportions of eggs laid on the two host plant species were significant in treatments B and C for both butterfly subspecies (Mann-Whitney: *O. o.* ssp. : Treatment B

: *U* = 31 p < 0.01; treatment C : *U* = 21 p < 0.01; *O. o. agarista* : Treatment B : *U* = 26.5 p < 0.01; treatment C : U = 17.5 p < 0.01) where *S. mite* was the most preferred plant (Fig. 8), whereas in treatment D the two subspecies laid eggs in similar proportions on leaves of both host plant species (*O. o.* ssp. : treatment D : *U* = 73 p = 0.555; *O. o. agarista* : treatment D : *U* = 72 p = 0.095). For *Solanum anceps* the proportion of eggs on leaves increased markedly with number of plants in the cage; however, *O. o.* ssp. showed a greatest preference for *S. mite*. The proportion of eggs on *S. mite* leaves was high for treatment A, but similar between treatments B and C, and fall drastically in treatment D for both subspecies. Nevertheless, *Solanum mite* was the most preferred host plant for both *Oleria* subspecies. *O. o. agarista.* appeared to accept more readily the host-plant switch, and showed a greater preference for *S. anceps* than *O. o.* ssp.

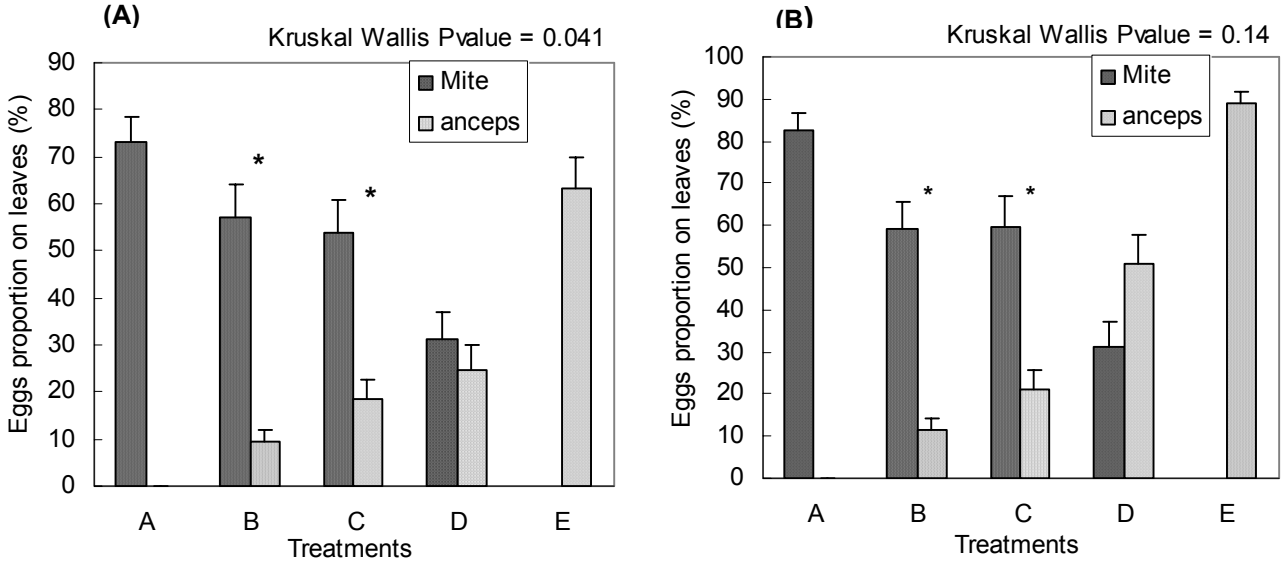

Figure 8 : Results from a choice test with different proportion of the two host plants in five treatments with (A) *O. o.* ssp. and (B) *O. o. agarista*

The difference between the proportion of eggs laid on the two host plants were significant for treatments B, and C, (* indicate the significant results) but not for treatment D (Mann-Whitney : **Fig 8A** : treatment B: : *U* = 31 p < 0.01; treatment C : *U* = 21 p < 0.01; and treatment D p > 0.5; **Fig 8B** : Treatment B : *U* = 26.5 p < 0.01; treatment C : U = 17.5 p < 0.01 and treatment D p > 0.09).

DISCUSSION

oviposition preferences

The results from the screening experiment on oviposition preference showed that the four presumed host plants were chosen by both subspecies of butterflies and, among these, *Solanum mite* was their most preferred host plant. In the experiment with a single female per cage, no eggs were laid on or near the control plants. It can thus be assumed that all eggs on the wall of the cage were laid after recognition of the nearest host plant (within a distance of 40 cm.). In the screening experiment, a large number of eggs was laid on *S. uleanum* by *O. o.* ssp. females; this may be due to chemicals similarities between this plant and *S. mite*. However, there might be another explanation: it has been demonstrated that when a female is more motivated, it is more likely to accept a non-preferred host plant for oviposition (Fitt, 1986, Singer et al., 1992, Hopkins & Van Loon, 2001). In our case, only one *S. mite* individual was present in the cage, with several females laying eggs on it. The high competition in this case may induce a highly motivated female to oviposit on a less accepted host. This remains a hypothesis, as the motivation (i.e. number of eggs in the abdomen) of the female was not defined.

The hierarchy in host preference is similar in both subspecies. They first choose Solanum mite, then Solanum anceps, and finally S. angustialatum and uleanum with no great difference. Nevertheless, it might be kept in mind that all the females used in these experiments came from the field, and were therefore not “naive”. In most oviposition preference studies, newly emerged and mated females are used (Craig et al. 2000; Bernays, 1999; Schöps & Hanski, 2001; Hopkins & Van Loon, 2001) but in our case no individuals born in the laboratory had accepted to mate. The results may be biased mostly for O. o. agarista that naturally have S. mite, S. anceps and S. uleanum in their environment, whereas O. o. ssp, only knows S. mite. However, learning appears not to have an effect on the results, as preferences of both Oleria subspecies were quite similar.

Differences in preference for leaves and other substrates.

The egg laying behaviour of the two *Oleria onega* subspecies indicated differences in the choice of oviposition sites, i.e. whether eggs were laid on host-plant leaves or on objects close by. *Oleria onega agarista* oviposited mostly on the leaves, whereas *O. o* ssp. searched for other substrates to lay eggs. When the eggs were laid on the host plant, no specialisation was observed on a particular site, as

occurs with other lepidopteran species (Benson 1978; Thompson 1983; Janzen 1988; Rausher 1979, Chew & Robbins, 1984; Higashiura 1989). The known reasons for a within-plant specialisation are climatic factors in cold regions (Higashiura 1989), protection against predators (Benson 1978; Thompson 1983; Janzen 1988; Rausher 1979, Chew & Robbins, 1984) and quality of food for the larvae (Barker & Maczka, 1996, Craig, 2000; Steiner & Trusch, 2000), even though female choice is not always correlated with influence of oviposition site on offspring fitness. In the case of *Oleria,* females can lay eggs on old leaves as well as on young meristems, stems and any other part of the plant. We may conclude that the site on the plant does not affect the larval performance, and are not chosen to avoid predators nor to provide the best food for the larvae. In contrast, oviposition on objects near the host-plant suggests a high selection pressure against eggs, reducing their survival when laid on any part of the plant. Costs seem to be higher for *O. o.* ssp., on the SW slope, as this behaviour was scarcely observed *in O. o. agarista*, coming from the NE side. We presume that the main factor of costs, inducing this behaviour is the presence of natural enemies of eggs and larvae, occurring more frequently on the SW slope. The behavioural difference between the two subspecies was greater when several females were in the same cage than in experiments with a single female per cage. This result suggests that the fact of not laying eggs on the host plant might be more accentuated when higher competition between females for oviposition sites is greater, and this may influence the behaviour and preferences of all females. It has been reported that, for some species that usually lay eggs in clusters, the size of the clutches decreases when the number of eggs-laying females per patch increases (Parker & Courtney, 1984; Parker & Begon, 1986). As density of female was higher on the SW side than on the NE side, the difficulty encountered by females in finding the best place can be compensated by a higher egg survivorship and the high host plant density. In our case, when a high concentration of females occurs, the existing behaviour of avoiding to oviposit on the host plant, may be influenced by a new cost, which is competition.

Effect of density and diversity of plants

In the experiment conducted to test the effect of density there were no effects on oviposition pattern but this result may be biased since females generally laid few eggs in this experiment, and these results will not be taken into account here.

In the experiment with different host plant species composition, we could expect that the choice will reduce the number of eggs laid in the mixed treatments, as observed in other study, due to the fact that complex stimuli reduce "decisiveness" or allow distraction (Bernays, 1999; Hopkins and Van Loon, 2001). In our case, when the two host plants were present, unexpectedly more eggs were laid than in the treatment with a single plant species. When only *S. anceps,* the host of low acceptability*,* was present the number of eggs laid was similar to that when only *S. mite* was present. This may be explained by the fact that when female have a "long" life time (*Oleria* live up to two months), they have the possibility to wait when conditions for oviposition are not optimal. In our case, females laid more eggs when they have the opportunity to choose the plants (in the mixed treatments). If they had a shorter life they may lay eggs in the same quantities in all the treatment. However this hypothesis is hard to apply in our case as we ignore the real age of the females used.

Therefore, even though *S. mite* was the most preferred host, a switch to other host plant (such as *S. anceps*) may occur easily. This was clearly demonstrated by the non-significant result of the Mann-Whitney test for treatment D for both butterfly subspecies. When one *S. anceps* was present with three *S. mite*, this single plant was neglected whereas when three *S. anceps* were present with one *S. mite* (treatment D), *S. mite* was still the more attractive (in the case of *O. o.* ssp.), but *S. anceps* was also accepted as a host plant When both host plants were present in equal proportion, *S. mite* was the most preferred by both butterfly subspecies. *O. o. agarista* seemed to accept *S. anceps* as a host plant readily than did *O. o.* ssp.. This is logical, as both butterfly and plant are found in the same environment in the field. The proportion of eggs laid on leaves was nearly the same for *O. o.* ssp. and *O. o. agarista* (Fig 8). Both subspecies apparently reacted similarly to the different composition of plants in the cages, although *O. o.* ssp. did not show as great difference in the choice of the oviposition site (on and away from the plant), as it did in the previous experiment. However it might be recorded that for this experiment fewer females of *O. o.* ssp. were tested than for the other subspecies. Another problem with this experiment was that few females were available, and therefore some of them were tested twice with the same plant proportions (but plant specimens were changed) and could then learn to recognise the plant species. However some studies have revealed that learning is greater in butterflies when foraging than when laying eggs (McNeely & Singer, 2001) and that sometimes butterflies do not learn after previous encounters with different host plant (Schöps & Hanski, 2001).

How do these results fit with the natural composition of plants and mortality due to predators?

We supposed that *Oleria onega* ssp.*,* which does not encounter *S. anceps* and *S. uleanum* in its natural environment, would neglect them more than did *O. o. agarista*. However preferences of both subspecies were similar. In some cases, *O. o.* ssp. showed a higher acceptability for new hosts. For example in the screening experiment, the high number of eggs laid on *S. uleanum* by *O. o.* ssp. females and could be related to the fact that some of them were highly stimulated to oviposit and that density of *S. mite* in the cage was too low to satisfy the female constraining it to choose a new host. Even if any switch to a new host involves genetical and learning parameters that take time to be acquired by a female (Singer, 1983, 1986; Jaenike 1990; Thompson 1988; Thompson & Pellmyr, 1991, Wiklund, 1975), it has been noted that in a specialist population, when the preferred host is scarce, the shift to a host of lower acceptability may be carried out quickly by some individuals that are more generalists, whereas the majority of individuals of the population retain egg loads in case a superior resource becomes available later (Wiklund, 1981, Hokins & Van Loon, 2001). In our case, a factor that could facilitate host switching is that in the four tested plants, the chemical compounds should be similar as the plants are closely related.

For the oviposition site preferences, we suppose that the main factor of costs inducing the behaviour of not laying eggs on the host plant is the presence of natural enemies of eggs and larvae, and eventually ovipositing females, occurring mostly on the SW slope (see Chapter 2). The climatic conditions around the host plant and the food quality factors do not seem to have such importance to induce a new oviposition strategy, however host plant are regularly cut by the farmers and this may be another factor inducing the female to avoid oviposition on the plant. As this behaviour was accentuated when many females were in the same cage, we suppose that this strategy, first induced by predation, may be used thereafter by the butterflies as a way to reduce competition. In contrast, a factor that may have helped the butterflies to develop this behaviour on the SW side, is the higher host-plant density observed there, making food easier to find for a newly hatched larva.

CONCLUSION

The most pertinent result found in this study was that both cage experiments and field observation revealed that the two butterfly subspecies have a distinct behaviour. *O. o.* ssp. lays most of its eggs on objects close to the plant and *O. o. agarista* on the plant itself. It is important to note, however, that in both cases, exceptions were observed. Another interesting result revealed by the experiments conducted in the cages, is that *Solanum mite* is the most preferred host plant, but that oviposition switches to other *Solanum* species occur easily when *S. mite* is rare or absent. Laying eggs away from the plant could be a new behaviour that *O. o.* ssp. is developing, but that not all females have already completely evolved. On the other hand a few *O. o. agarista* females seem to adopt this behaviour. A possible explanation may be that for *O. o.* ssp. the costs due to predation on the host plant induced the butterflies to find a new strategy, searching for the more secure place for the eggs. The cost of oviposition away from the plant may be compensated by less competition for the ovipositing females to find a place on the plant, and by the higher host plant density found on the SW side of the mountain. The composition of predators should be different between both mountain sides, and one (or several) predator species are not found on the NE slope. A second hypothesis, completely different, may be that in the SW slope, there are more farmers and more deforestation, since at least fifteen years. The large patches of *Solanum mite* found on the path-hedges, are regularly cut to clean the path. As small plants are still present after cutting, it is possible that the butterflies have developed the behaviour of not laying eggs on plants, hence allowing greater chances for the larvae to find their food plants. This adaptation is possible due to high number of butterflies generations per year (at least six generations) and due to the fact that deforestation became greater from fifteen years ago, that may "learn" to avoid oviposition on endangered sites.

New questions arise from this study that we will partly be tried to answer in Chapter 2: how suitable are the four host plants for the larvae? Are there correlations between female preferences, and larval preference and performance? Are soil fauna and presence of potential predators on plants different between both mountain sides? Are there inducing heavy costs, enough to a new behaviour to arise?

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Chapter 2

Comparison of larval behaviour of two Oleria onega subspecies (Ithomiinae, Lepidoptera), in relation to predators of their early stages.

Based on:

Gallusser, S.; Rahier, M.

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INTRODUCTION

In theory, the successful development of the larvae should be the main factor dictating the female oviposition choice however, in nature, other environmental factors are also known or suspected to play an important role, such as: i) availability of resources for adults (food and mating sites), ii) factors influencing temperature and/or light intensity (shady versus open sites), iii) distribution of predators with respect to potential host plants, iv) plant morphological characteristics such as size or shape which may be correlated with larval success, and v) density and dispersion of host plants. In some cases females oviposit in places that are not advantageous for their offspring (Rausher 1979, Chew & Robbins, 1984, Higashiura, 1989). Indeed, numerous studies have compared the preferences of ovipositing females for certain host plants with the performance of the larvae, revealing that in most of the cases this correlation is unexpectedly low (Williams, 1983; Pires et al., 2000; Craig et al., 2000, Harris et al., 2000). In exchange, other cases have shown that correlation may be very high (Nylin & Janz, 1993; Barker & Maczka, 1996). However, study on host preferences can lead to a confusion as discrimination within host species can mask or confound discrimination among species (Singer & Lee, 2000). For both female preference and larval performance, we can observe a selection among the plants chosen by the females and those that the larvae are favoured, and ranking of host plant is not always the same for females and their offspring (Harris et al., 2001). Many factors may interact to determine preferences and performances of both gravid females and their offspring, and a failure to consider important factors, or the interactions among factors, may lead to an incomplete understanding of the preference-performance relationship. However, discrepancies may also be due to conflicting selection pressures acting on ovipositing females, with for example, increased predation on higher quality hosts leading to a lack of directional selection (Clancy, et al. 1993), or to avoidance of oviposition on the plant (Inouye & Taylor, 1979; Freitas & Oliveira, 1996).

Some butterflies are known not to oviposit on their host plants. However this particularity has been observed mostly in species that overwinter in the egg stage and whose host plants are superabundant (Wiklund, 1984). In the tropics, the particularity of ovipositing on objects near the host plant has not yet been observed. In contrast to the observed cases in temperate regions, predation is likely to be one of the possible factors that may induce this behaviour as environmental factors such as temperature and humidity do not considerably vary in time and space at a small scale in a tropical

forest. Previous studies on ants have shown that predation on larvae may be reduced by larval defence mechanisms but also by behaviour of the gravid female (Inouye & Taylor, 1979; Freitas & Oliveira, 1996). Larval defences may involve scoli, spines and hairs (Frost, 1959), unpalatability (Brower, 1984) camouflage and mimicry (Edmunds, 1974), myrmecophily (DeVries, 1991) and the construction of frass chains (Machado & Freitas, 2001). In contrast, survival of the egg stage may not depend exclusively on intrinsic egg protection factors (unpalatability, camouflage) but also on female oviposition behaviour.

The aim of this study is to investigate the larval biology of two Ithomiinae subspecies that are endemic to north-eastern Peru: *Oleria onega agarista* (Felber & Felber) and *Oleria onega* ssp. a recently discovered subspecies. Interest in Ithomiinae was first focused on their mimetic particularities (Bates 1962, Müller 1976, Brown & Vasconcellos-Neto, 1976; Brown, 1979) and on their chemical relations with plants (Brown, 1984 a, b,). Relations between Ithomiinae and their larval host plants were described at the genus level by Brown (Brown, 1987) who showed that the genus *Oleria* is associated with *Solanum* species. However, the larval biology of the different species remain patchily studied and that only a few of species have been described in more detail by Young (Young, 1972, 1973, 1974a,b,c, 1977, 1978a,b) and by Haber (1978). More recently, juvenile stages of most species of the genus were described and photographed by Brown (Brown & Freitas, 1994). Field observations in the north-eastern Peru, near the town of Tarapoto (S 06°22'50'; W 076°26'23''), suggested that four *Solanum* species are probably used by *Oleria onega* subspecies as host plants (Drummond & Brown, 1987; Knapp & Helgason, 1997): *S. mite* (Ruiz & Pav.), *S. anceps* (Ruiz & Pav.), *S. angustialatum* (Bitter) and *S. uleanum* (Bitter). The “Cerro Escalera” is a mountain chain which constitutes a strong biological barrier between areas with considerably different fauna and flora (Joron 2000; Joron et al., 2001; Schulte 1999, Mallet, 1989, 1993). *O. o.* ssp. is mostly found on the south-western slope, whereas *O. o. agarista* occurs on the north-eastern slope (abbreviated below as SW and NE slopes) and in the Huallaga Valley. Both subspecies occur at 200 - 800m altitude. On the NE slope, three of the four Solanaceae species occur in sympatry: *S. mite*, *S. anceps*, and *S. uleanum*, whereas on the SW slope only *S. mite* is found. *S. angustialatum* grows on the upper part of the mountain (≈1'100 m alt.). In the habitat of *O. o.* ssp., where only *Solanum mite* is found, the abundance of this host-plant (i.e. density and area covered) is higher than on the other side of the mountain. *S. mite* grows abundantly in secondary vegetation after deforestation, which is greater on the SW slope. Therefore open areas are greater in size and allow for bigger plant patches, mostly on the path edges (Knapp & Helgason, 1997).

Female oviposition preferences were tested in a previous study (Chapter 1) that revealed a hierarchy among the four plants: *S. mite*, the most abundant and the most preferred host, followed by *S. anceps* and subsequently by *S. angustialatum* and *S. uleanum,* which do not differ in their attractiveness. Nevertheless the influence of different plants on larval fitness remains unknown. Females of the genus *Oleria* generally lay their eggs singly on the underside of the leaves of their host plant. However, *Oleria onega* ssp. females showed the particularity of laying most of their eggs on objects near rather than on the host plant (Chapter 1). After successful recognition of the host plant, females flew at most one meter away from the host plant and subsequently laid their eggs on stems, dead plant material, rocks or other plants (randomly chosen by the female). In any case the support on which the eggs were laid was never found to be higher than the host plant. In contrast, females of *Oleria onega agarista* lay most of their eggs on the host plant. As animal and plant communities differ in composition on the two sides of the mountain, we hypothesise that the distinctive behaviour of laying eggs next to the host plant may be correlated with the presence of egg predators, as eggs might be easier to find when laid on leaves of the host plant than when laid on other substrates.

The questions addressed here are:

Is female preference for S. mite positively correlated with larval preference and performance?

Do O. o. ssp. females lay their eggs in the vicinity of the host plant, instead of on them, to avoid predation?

Do egg survival rates differ between the two Oleria subspecies?

Do larval activities during the day (and night) differ between the two subspecies in ways that might result in avoidance of predation events?

How different is the soil surface fauna, and therefore the potential guild of natural enemies, between the NE and SW slopes of the mountain?

Are some natural enemies present on the SW but not on the NE slope?

As the biology of Oleria remains unknown, the different developmental stages will be described and the duration of each detailed

MATERIALS AND METHODS

Study organisms. *Oleria onega agarista* (C. Felder and R. Felder) and *Oleria onega* ssp. nov. are two closely related subspecies endemic to north-eastern Peru. *O. o. ssp* differs morphologically from *O. o. agarista* by the narrower black edge of the wings and by to black bands on the hindwing that are never connected (Lamas, pers. comm.). The subspecies *agarista* is characterised by a transversal band connecting these two bands on their middle part on the Cu1 and Cu2 veins. The morphological hybrids are recognisable by an incomplete transversal band. The butterflies used in this study came from populations at the Shilcayo and Ahuashiyacu sites for *O. o.* ssp., and from Km30 in case of *O. o. agarista*. The potential host plants that we tested here all belong to Solanaceae family and the section Pteroidea of the genus *Solanum* (Knapp, 1997). Three of them, *Solanum mite*, *Solanum anceps* and *Solanum angustialatum* are single-stemmed herbs that grow to a height of max. 1.5 m whereas the fourth species, *Solanum uleanum*, is a small vine. The specimens of *S. anceps* and *S. uleanum* used for feeding and for oviposition experiments in cages came from the Km30 site, *S. angustialatum* was collected near the Tunnel (near the top of the mountain), and *S. mite* at Shilcayo.

*Study site*s: All fieldwork was carried in the north-eastern Peru, on the flanks of the mountain "Cerro Escalera". The study sites are situated between the town of Tarapoto (SW slope) and Km30 of the road (Carretera marginal) to Yurimaguas (NE slopes) (S 06°24- 06°28 and W 76°18 – 76°22). The road crosses the Cerro Escalera mountain through a tunnel (alt. ≈ 1100 m). The two sides of the mountain contrast in their climatic conditions: the SW side is usually sunny and hot (mean temp. ca. 30°C), while on the other side the climate is wet and rainy including fog and cooler temperatures (mean temp. ca. 27° C). The Shilcayo and Ahuashiyacu sites are located on the SW slope, whereas the Km30 site is situated on the NE side.

For laboratory experiment, the butterflies were kept in cages made of wood and wire-mesh (plastic and metallic) that was stapled to the wood. To reduce mortality due to ants, the cages were standing raised above the ground. Cage size was $1m^3$, allowing the butterflies to fly. Constant access to small recipients with 10% sucrose solution was insured in the cage. *Heliotropium* flowers were regularly put into the cage as a source of pyrrolizidine alkaloids (PA's) (used for chemical defence and as pheromons precursors) as well as bird droppings as an amino acid source for egg production.

Immature development larval preference and performance on different host plant species.

Five to fifteen individuals of each host plant species were collected and potted to facilitate their manipulation. Females of *O. o.* ssp. and *O. o. agarista* were separated into two cages, with 10-15 females per cage. One individual of one of the four plant species (*S. mite*, *S. anceps*, *S. angustialatum* and *S. uleanum*) was placed in each corner of the cage. Plants were exchanged or rotated daily in cages, so that each day females were offered a new situation. Every second day all eggs laid were collected and it was noted on which host plant they were laid. Eggs encountered on other substrates within 40 cm from a plant were considered as associated with this plant.

A total of 120 individuals were reared from the egg to the adult stage on *Solanum mite.* The duration of each stage was noted for both subspecies.

A total of 273 eggs laid in the cages by both subspecies were placed individually in the center of Petri dishes with a piece of leaf of each of the four host plants around them. Just after hatching, the first host plant that was fed upon by each larva was recorded and considered as preferred. We tested the significance of correlation between larval preferences and female oviposition preference (see Chapter 1).

A further experiment was carried out with 130 eggs of both *Oleria* subspecies to compare larval performance on the two most common host plants, *S. mite* and *S. anceps*. Each larva was fed from hatching to pupation with the same plant species. Three parameters were taken into account to test the suitability of the different plant species: larval mortality, duration of larval development and three morphological measurements of pupal size (the length and two widths).

The survival of each subspecies on the two host plant was compared using Fisher's exact tests. To compare the duration of larval and pupal development and the size of pupae of each subspecies on the two plants, we used a general linear model ANOVA procedure for unbalanced data (Sokal & Rohlf, 1995).

Egg survival in the field

To estimate egg mortality rates due to natural enemies in the field and to compare these between the two slopes of the mountain, a total of 400 eggs laid in cages were glued on plants of the two main study places: Shilcayo (SW side) and Km30 (NE side). In each site 50 eggs of each subspecies were glued on the underside of *S. mite* leaves and 50 others on stems of other plants,

stones, sticks, dead leaves etc. (50 *O. o.* ssp. + 50 *O. o. agarista* on leaves + 50 *O. o.* ssp. + 50 *O. o. agarista* on other substrates x 2 sites = 400 eggs). As entomological glue is not resistant to rain we used normal universal glue. To avoid direct contact of the eggs with the glue, a small part of the support (plastic bags or on *S. mite* leaf) on which the egg has been laid was cut out. Eggs were exposed the day they were laid. After two days in the field they were collected again to avoid their hatching during the experiment. The number of eggs still present was counted and each egg was then put into a small vial to study eventual parasitisation. At each site the whole experiment was performed within one week. Differences in egg survival rate between the two subspecies in each site were analysed pairwise for the same support (leaves or other substrates) by Fisher's exact tests. Differences between egg survival rate on leaves and on other substrates were also analysed. Finally differences between the two sites (Km30 and Shilcayo) were compared. The eggs that had hatched were considered as having survival. Succesfully hatched eggs are usually easy to recognise because the larvae almost always leave the part of the eggshell glued by the female on the leaves, although the rest of the shell is eaten.

The second part of the egg survival experiment was carried out only at the Shilcayo site where predation was expected to be higher. Only *O. o.* ssp. eggs were exposed. Fifty eggs were glued onto each of fifty *S. mite* plants. Half of the plants were treated with protecting sticky glue around the stem, thus "walking" predators were excluded. Eggs were re-collected and counted after two days. Results were analysed with a Fisher's exact test between the two treatments.

Larval activity

A total of forty-one larvae of the two subspecies (*O. o.* ssp. from Ahuashiyacu and *O. o. agarista* from Km30), were placed singly into vials to compare their circadian activity patterns. Larval activity may have been affected by differential selection pressures related to predation. All larvae were reared from the egg stage. Fresh leaves of *S. mite* were provided daily. During a period of 31 days larval activity was checked four times daily: at 07.00h., 12.00h., 17.00h. and 22.00h.. Activities were classified as: resting, walking, eating, moulting, and pupating. A factorial analysis was performed using the three main activities: resting, walking, and eating, to compare the differences between the two subspecies. In a second analysis, walking and eating were grouped, as being the activities during which larvae are more exposed to natural enemies.

Predators

From October to February four malaise traps and ten Barber traps were installed once a month on each mountain slopes. They were left for five days in the two main sites Km30 (NE slope) and Shilcayo (SW slope). Arthropods collected were identified to family level for all of them, and additionally to genus level for ants. The diversity of families (for insects) and order for (spiders and harvestmen), and arthropod abundance, were compared between the two sites. Ants were collected manually at both sites to test their potential role as egg predators in the laboratory. At the Shilcayo site where predation was expected to be higher, ants were also collected during the night. A patch of twenty plants (host and non-host) was checked during one hour and all the ants on them were collected with an entomological aspirator and identified to genus level. At Km30 five hours of collection were performed because of the less accessibility of the site, whereas at Shilcayo twenty hours were carried out during the day and three hours during the night. Host plants with eggs on their leaves were potted and the pots were sealed with paper lids to hinder ants from entering the soil in the pot. Ants were placed individually on the paper lid and the behaviour of each ant was observed for 15 minutes. It was recorded whether each ant climbed onto the plant, approached the eggs and whether any eggs were eaten. At least five individuals of each ant species were tested, less abundant species with fewer than five individuals were discarded. The behaviour of the ants when placed on the paper was noted. The species that showed an aptitude to climb quickly on the plant were re-tested with at least twenty individuals.

RESULTS

Immature development, larval preference, and performance on different host plant species.

Eggs are small, oval and white, with small features on the surface. Eggs are always laid singly. The egg stage lasts an average of three day. After hatching, the transparent larva first eats the eggshell before feeding on the host plant leaves. Larvae become grey with one yellow stripers on each side of the body. *Oleria* (and all Ithomiinae) have five larval instars, and at each moulting they eat the old skin and leave only the sclerified head capsule; they moult every two or three days. After two weeks the caterpillar stops eating, becomes green and begins pupation The time lapse between the final feeding and pupation usually lasts about twelve hours, but in some cases the larvae can stay more than twenty-

four hours, perhaps waiting for appropriate conditions. The pupae is a beautiful green drop, with shiny golden metallic reflects. There is some colour varieties (brownish or yellowish) in the same subspecies, but this polymorphism did not affects the adult coloration. After 6 days the future wings become black with white dots and the wing pattern of the imago can already be seen. After 7 or 8 days the butterfly ecloses, drying its wings within one hour. Eclosion can take place at any time of the day.

Figure 1 shows the mean duration of each stage and the total duration of the larval (five instars) and pupal stages. The results were obtained from 120 individuals from each of the two subspecies, fed with *S. mite*. The durations of the larval stage differed slightly but significantly between *O. o.* ssp. and *O. o. agarista*, with *O. o. agarista* showing a slightly shorter duration of larval development (Fig 2) (*t*-test p-value= 0.003). Egg development took on average three days while larvae needed twelve days to finish their development. The mean duration for the pupal stage was seven days. Standard errors were very low, revealing little variation between individuals.

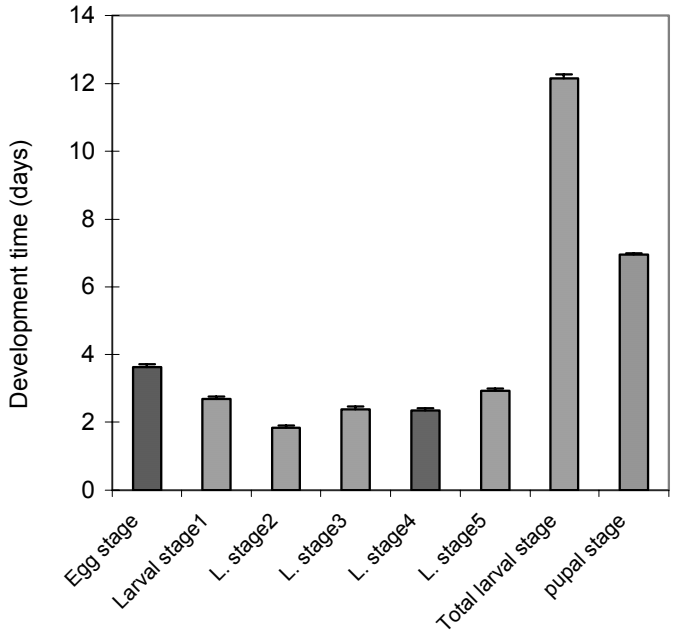

Fig. 1 : Results from observation of growth of immature stages of 120 butterflies of the two *Oleria* subspecies, reared on *Solanum mite*.
Bars indicate the duration of the different stages : the eggs, the five larval stages separately, the whole larval stage, and the pupae.

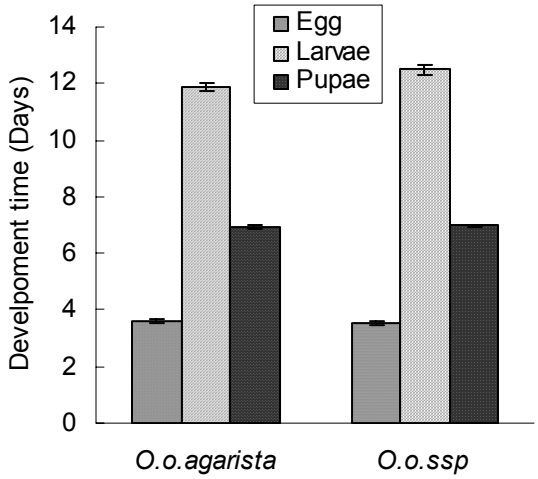

Fig. 2 : Comparison of duration of development time between *O. o.* ssp and *O. o. agarista*.
Bars indicate the three different stages of the two subspecies. Larval development time was significantly longer for *O. o.* ssp. (T-test : p= eggs 0.78, larvae 0.0038, pupae 0.666).

The correlation between female oviposition preferences and larval feeding preferences is shown in figure 3. A total of 273 eggs were laid by the females of *Oleria onega* ssp. and *agarista* on the four host plant. In both butterflies subspecies no differences were found between preferences of females and larvae (result not reported here). Therefore data of the two subspecies are combined in this analysis. Results revealed that *S. mite* is the host plant most preferred by both the females and the larvae followed by *S. anceps.* The two species *S. angustialatum* and *S. uleanum* may be considered as hosts but attraction to both plants is relatively low.

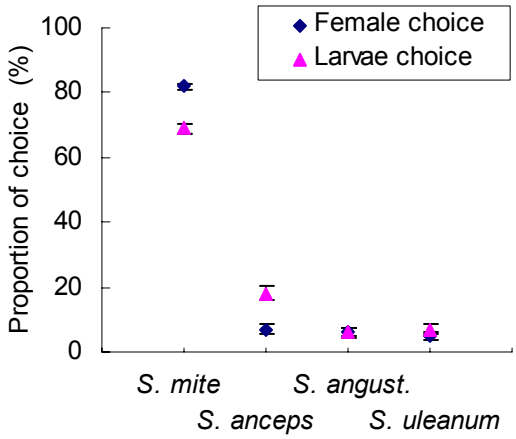

Figure 3 : Comparison of female and larval host plant preferences (N= 273 eggs).
Females and larvae were offered four host plants, *S. mite, S. anceps, S. angustialatum* and *S. uleanum*. For both female and larvae, data for both subspecies were analysed together, as no differences were observed between them.

Larval performance was tested on the two most preferred host plants, *S. mite* and *S. anceps,* with a total of 130 eggs from both subspecies (116 *O. o.* ssp. + 14 *O. o. agarista*). High mortality rates were observed as only 65 individuals reached the pupal stage. The individuals that have reached the pupal stage give the survival percentages (table 1) No differences in survival rates between the two subspecies were found for either two host plants (Fisher's exact test: $p > 0.4$ for both plants). Differences of survival between the two host plants were significant for *O. o.* ssp. with a better survival on *S. mite* (Fisher's exact test: $p = 0.0028$) but not for *O. o. agarista* (Fisher's exact test: $p > 0.5$), Analysis of variance (ANOVA) of larval development time of each butterfly subspecies on the two host plants showed that there was no significant effect of the butterfly subspecies nor of the plant species (between butterflies: $F = 0.21$, d.f. = 1, $P > 0.5$; between plants: $F = 1.14$, d.f. = 1, $P > 0.2$). The pupal, development time was slightly longer for larvae that have been fed with *S. anceps* (between plants: $F = 3.99$, d.f. = 1, $P = 0.04$), and the two subspecies did not differ in this aspect (between butterflies: $F = 0.09$, d.f. = 1, $P > 0.5$) (Table 1). Length of pupal was significantly different between the two subspecies when reared on *S. mite* ($F = 16.67$, d.f. = 1, $P = 0.000$). However, a host plant effect was also observable since pupae of both *Oleria* subspecies were smaller when reared on *S. anceps* ($F = 63$, d.f. = 1, $P = 0.000$).

Table 1 : results of the development comparison of the two *Oleria* subspecies on two host plants : *S. mite* and *S. anceps*. Four parameters were taken into account: the larval development time (larvdevt), the pupal devevlopment time (pupdevt); the pupal length (Pupal L) and the proportion of individuals that reach adult stage (survival).

	S. mite				***S.anceps***			
	larvdevt (days)	**pupdevt (days)**	**Pupal L. (cm)**	**Survival (%)**	**larvdevt (days)**	**pupdevt (days)**	**Pupal L. (cm)**	**Survival (%)**
O. o. ssp.	17.26 (± 0.21)	7.08 (± 0.10)	10.89 (± 0. 07)	67 (± 0.03)	16.95 (± 0.41)	7.32 (± 0.10)	10.01 (± 0.11)	38 (± 0.03)
O. o. agarista	17.33 (± 0.88)	6.83 (± 0.40)	10.29 (± 0.11)	86 (± 0.05)	16.25 (± 0.25)	7.50 (± 0.28)	9.44 (± 0.21)	57 (± 0.09)

Larvdevt = larval devevlopment time; pupdevt = pupal devevlopment time; Pupal L. = pupal length; survival= percent of individuals that reach adult stage

Eggs survival in the field

For this experiment 100 eggs of each butterfly subspecies were glued onto host plants (half of the eggs) and other objects (half of the eggs) on the SW slope (Shilcayo) and the same was done on the NE slope (Km30). No significant differences were noted between both subspecies with respect to egg survival. (Fisher's exact test : p> 0.5 for all), therefore the results for the two subspecies were pooled for the following analyses. Differences between survival rates on leaves and on other substrates were analysed for each site. In Shilcayo a significantly lower survival rate was found for eggs laid on host plant leaves compared with eggs laid on other substrates (Fisher's exact test: p = 0.000) (Fig.4A). On the other hand, no differences in egg survival were noted between eggs laid on host plant leaves and those laid on other substrates at the Km30 site (Fisher's exact test: p > 0.3) (Fig.4B). When comparing the survival of eggs of each subspecies exposed on the same substrate but at different sites it became evident that more eggs on leaves were missing in Shilcayo than at Km30 (Fisher's exact test: p = 0.02). No differences in survival rate were found between the two sites for those eggs laid on objects (Fisher's exact test: p > 0.3)

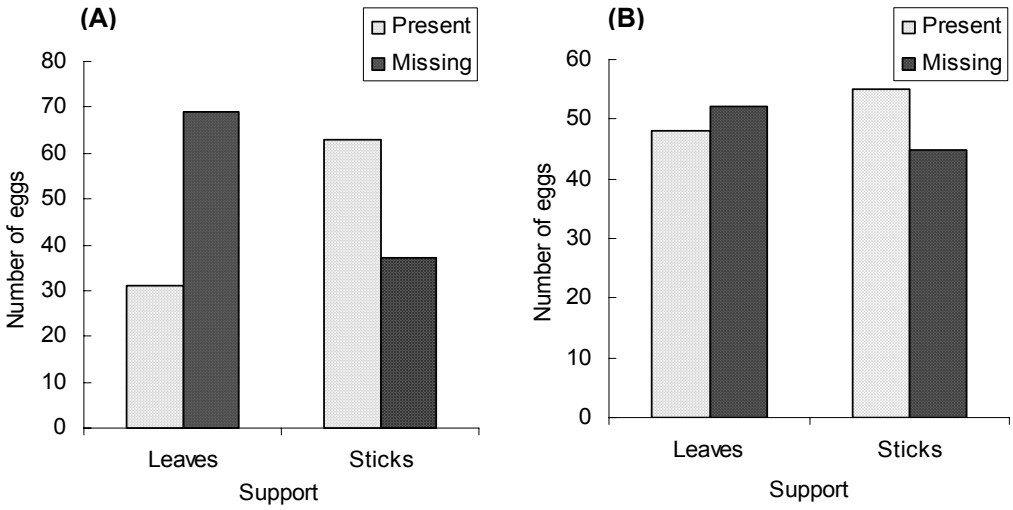

Fig 4 : Survivorship of eggs of two *Oleria* subspecies on different substrates in two sites : (A) Shilcayo (SW slope) and (B) Km30 (NE slope).

Bars indicate the number of eggs present and absent after two days glued on leaves or on other objects. Data of both *Oleria* subspecies were analysed together, as no significant differences were observed between them. Egg survival was significantly higher when laid on other substrates in the Shilcayo site (Fisher's exact test: : p= 0.000), whereas no differences in survivorship were observed between eggs laid on leaves and on other substrates in the Km30 site (Fisher's exact test: p> 0.3).

On plants protected by sticky glue (figure 5) egg survival was significantly greater than on non-protected plants (Fisher's exact test: p= 0.022).

None of the eggs that were re-collected and placed in vials after the experiment were infested by parasitoids.

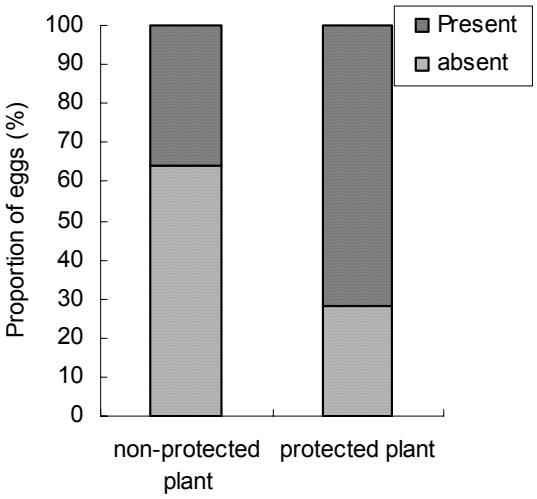

Fig 5 : Survivorship of *O. o.* ssp eggs glued on *Solanum mite* leaves in Shilcayo, with and without sticky glue on the stem of the host plant.

Bars indicate the proportion of eggs present and absent after two days in the field. Differences between the two treatements were significant (Fisher's exact test: : p= 0.02)

Larval activity

Preliminary interpretation of the data revealed that only three activities were frequent: resting, eating and walking. Larval activity was estimated for each subspecies (Figure 6). Our results revealed that *O. o. agarista* larvae move around more than do larvae of *O. o.* ssp. At 7.00 a.m. both subspecies showed little activity (resting). Between 17.00 and 22.00 larvae of *O. o.* ssp. showed a tendency to feed more, whereas at that time *O. o. agarista* larvae are mostly walking. As walking and eating were both activities where larvae are exposed to a higher predation risk, these two behavioural elements were grouped to form the category “activity”. A second factorial analysis was performed with the categories “rest” and “activity” (Figure 7). *O. o.* ssp. larvae showed a tendency to rest more than *O. o.* ssp. larvae. In general, both tended to rest more at 7.00h than at other time. At noon *O. o. agarista* larvae were more active than *O. o.* ssp. larvae. However, in the afternoon and at night *O. o. agarista* larvae reduced their activity, whereas the contrary was the case for *O. o.* ssp. larvae.

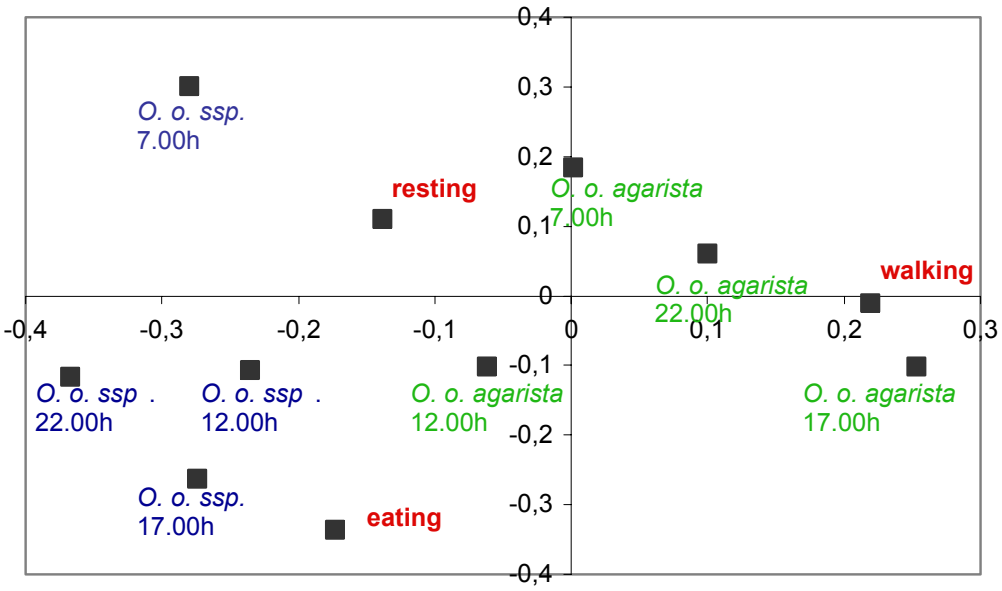

Figure 6 : Principal component analysis of the three main activities of the larvae of the two subspecies : *O. o.* ssp. and *O. o. agarista*

Eating, walking and resting were the three main activities. Squares indicate the positions of the activities and the positions of the different subspecies at four time : 7.00h, 12.00h, 17.00h, 22.00h.

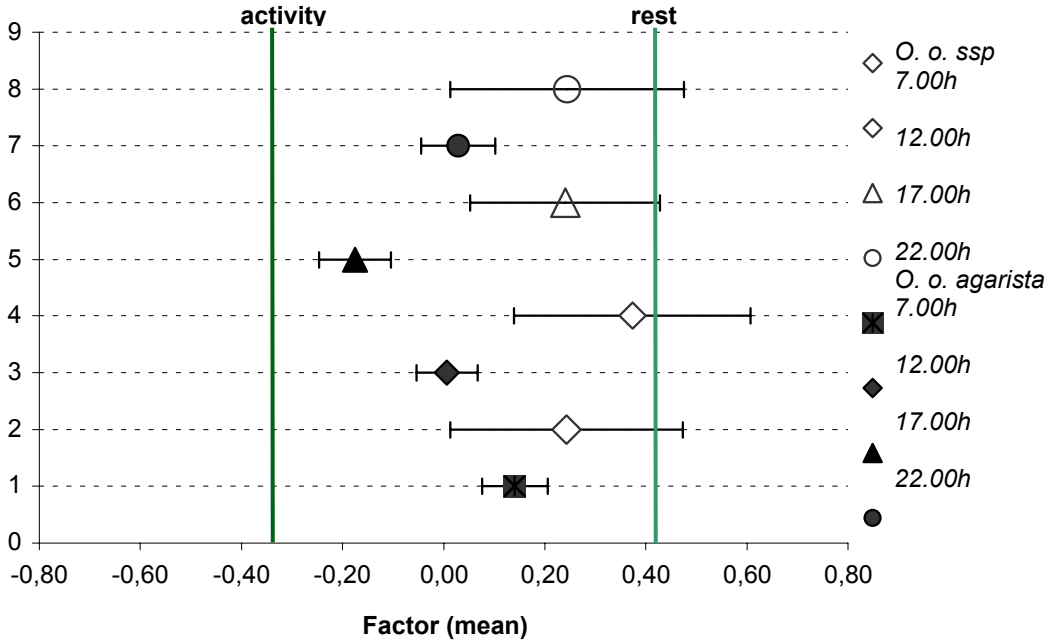

Figure 7 : Principal component analysis of the two larval states, active and resting, of the two subspecies : *O. o*. ssp and *O. o. agarista*

Activity and resting are the two states that occur in larvae. Black symbols indicate the positions of the *O. o. agarista* larvaes between the two states and the empty symbols those of *O. o.* ssp. at four different hours during the day: 7.00h, 12.00h, 17.00h, 22.00h.

Predators

Qualitative and quantitative differences in the composition of the fauna of the potential predator between the two slopes were assessed in five different months (Table 2). Ants and Gryllidae were much more abundant in Shilcayo (SW slope) than at Km30 (NE slope).

Table 2 : list of the potential predator groups caught with "Barber" and "Malaise" traps, in the two sites : Shilcayo (SW slope) and Km30 (NE slope)

Shilcayo						**Km30**					
Malaise traps	*Number of individuals caught*						*Number of individuals caught*				
	oct	nov	dec	jan	feb		oct	nov	dec	jan	feb
Araneae	0	1	1	1	2	Blattodea	3	1	0	0	0
Blattodea	4	3	3	0	1	Gryllidae	1	1	0	0	0
Gryllidae	3	1	0	0	1	Formicidae	12	10	2	4	5
Reduviidae	0	0	4	0	0	Staphylinidae	1	0	1	0	0
Formicidae	3	15	83	8	11	Cicindelidae	0	0	1	0	0
Carabidae	0	0	1	0	2						
Barber traps	*Number of individuals caught*						*Number of individuals caught*				
	oct	nov	dec	jan	feb		oct	nov	dec	jan	feb
Araneae	2	5	14	16	7	Araneae	4	3	6	4	3
Opiliones	1	1	1	6	1	Opiliones	4	6	3	0	5
Blattodea	9	3	18	2	9	Blattodea	2	2	1	1	9
Gryllidae	16	24	66	67	58	Gryllidae	12	9	2	3	9
Formicidae	25	76	538	126	224	Formicidae	67	78	23	73	99
Staphylinidae	3	1	18	5	7	Staphylinidae	6	4	5	1	4
Histeridae	3	0	2	0	1	Histeridae	0	1	1	1	1
Reduvidae	2	0	2	0	1						

Three genera of the predatory ants of subfamily ponerinea were found in Shilcayo but not at Km30 (Table 3). These were *Ectatomma, Hypoponera* and *Odontomachus*. However, only few individuals of the latter two genera were found. At Km 30, another ponerinea genus, *Prionopelta*, was found that does not occur in Shilcayo, but only one individual was caught. However three genera of the predatory subfamily Ecitoninae (*Eciton*, *Labidus* and *Neivamyrmex)* were recorded in greater abundance in Km30.

Table 3 : Ant genera caught with Barber and Malaise traps in the two sites : Shilcayo (SW slope) and Km30 (NE slope). Nb sp = number of species per genus, and food = alimentation. habits : p = predators, o = omnivorous; n= nectaries; c= fungi

Shilcayo			Km30		
Genus	**Nb sp.**	**Food**	**Genus**	**Nb sp.**	**Food**
Formicinae			*Ecitoninae*		
Camponotus	4	n	Eciton	1	p
Paratrechina	2	p	Labidus	1	p
Myrmicinae			Neivamyrmex	1	p
Crematogaster	2	o	*Formicinae*		
Acromyrmex	1	c	Camponotus	1	n
Cephalotes	1	n	Myrmelachista	1	n
Mycocepurus	1	c	Paratrechinae	1	p
Pheidole	5	o	*Myrmicinae*		
Solenopsis	2	o	Cephalotes	1	n
Ponerinae			Megalomyrmex	1	o
Ectatomma	2	p	Monomorium	1	o
Hypoponera	1	p	Ochetomyrmex	1	o
Odontomachus	1	p	Pheidole	3	o
Pachycondyla	3	p	Solenopsis	1	o
			Wasmannia	1	o
			Ponerinae		
			Pachycondyla	3	p
			Prionopelta	1	p

In Shilcayo, fifteen ant species were manually collected in the field using an aspirator. Their aptitudes to climb on plants and their interest in eggs were tested in the laboratory. At Km30 we collected twenty-three species, but only nine were abundant (Table 4). Among the sixteen species caught in Shilcayo (Table 4) six climbed on the plants. Five species belonged to the predator genus *Ectatomma*, and one to the omnivorous genus *Crematogaster.* For each of the six species their interest and their ability to eat the eggs were tested in the laboratory with twenty to thirty individuals (depending on the availability of the species). In these experiment, none of these species ate eggs of *Oleria* and their interest in eggs was generally low. *Odontomachus sp.* was not tested as only four individuals were caught and only at night. No individual of *Hypoponera sp.* was caught. *Ectatomma* species were more active during the day, but were also easily captured at night, as they rest on plants. This genus was present only on the SW slope. The ants of the NE slopes were only tested with five to ten individuals

because of the few availability of ants. No further observations were done on them, as no special oviposition behaviour was observed there on *Oleria onega agarista*. Genera including potential predator on the NE slope were mostly *Eciton* and *Camponotus*. *Camponotus* species were also found at Km30. *Eciton* species are army ants that attack other ant colonies, but also eat other arthropods.

Table 4 : Observation of ant behaviour when artificially placed near a host plant with eggs: food habits, their aptitude to climb, their reaction if put on the leaves (stay or go down), the estimation of the main behaviour (running, leaves inspection or grooming) their interest in *Oleria* eggs and an estimation of their density. Symbols for all the columns excluding density : - = not observed; + observed; ++ = observed very frequently. Symbols for density: - = 5 individuals caught, + = 5-10 individuals caught; ++ = 10-40 individuals caught.

	Alim.	climb on the plant	stay if put on the plant	Running	Inspect. of the leaves	grooming	taste eggs	High density in the field
Shilcayo								
Formicinae								
Camponotus sp.A	n	-	+	-	+	+	-	++
Camponotus sp.B	n	-	+	+	+	+	-	-
Formica sp.A	o	-	+	-	+	+	-	+
Myrmicinae								
Cardyocondila	o	-	-	+	-	-	-	-
Crematogaster sp.A	o	+	+	+	-	-	-	++
Dendromyrmex	o	-	+	+	+	-	-	+
Eucryptocerus	o	-	-	+	-	-	-	+
Pheidole sp.A	o	-	-	+	-	-	-	++
Pheidole sp.B	o	-	+	+	+	-	-	++
Ponerinae								
Ectatomma sp.A	p	++	+	-	++	+	-	++
Ectatomma sp.B	p	-	+	-	+	+	-	+
Ectatomma sp.C	p	+	+	-	+	+	-	+
Ectatomma sp.D	p	+	+	-	+	++	-	+
Ectatomma tuberculatum	p	+	+	+	+	-	-	+
Ectatomma sp	p	+	+	-	+	+	-	++
Odontomachus	p	-	+	+	-	+	-	-
Km30								
Ecitoninae								
Neivamyrmex	o	+	+	+	+	-	+	-
Formicinae								
Formica sp.B	o	-	+	-	+	+	-	+
Formica (Camponotus?)	o	-	+	-	+	+	-	++
Myrmicinae								
Crematogaster sp.B	o	+	+	-	+	+	-	+
Crematogaster sp.C	o	+	+	+	-	-	-	+
Megalomyrmex	o	-	-	+	-	+	-	-
Pheidole sp.C	o	-	-	-	+	+	-	+
Pheidole sp.D	o	-	-	+	-	-	-	+
Pseudomyrmicinae								
Pseudomyrmex	o	+	+	+	-	-	-	+

DISCUSSION AND CONCLUSION

Biology and performance of the larvae

The study on the duration of each immature stage has revealed that the egg stage lasted on average three days. Either of the five larval stages varied between two or three days in length. The first and fifth larval stages were slightly longer in time than stages two to four. The longest stage was pupation, which lasted seven to eight days. In the first observations on immature development time, duration of egg and pupal stages were not significantly different between the two subspecies. However, duration of larval development was one day longer (fig. 2) in *Oleria onega* ssp. This result was unexpected as we assumed that selection pressures by natural enemies was higher on the SW side, to which *O. o.* ssp. is restricted. As a consequence, the development of *O. o.* ssp. should be as short as possible to avoid the danger of being killed. However, in the experiment comparing performances on the two host plants *Solanum mite* and *S. anceps* (Table 1), the larval stage was longer than in the first observation on immature stage development (Figure 1). This was probably due to more favourable temperature conditions in the second experiment (humidity and temperature). Therefore, a realistic estimate of the duration of the larval stage in the field is probably between twelve and seventeen days, depending on temperature and humidity.

Among the four host plants tested, *S. mite* was the one most preferred by females in terms of oviposition choice (Chapter 1) as well as by larvae in terms of food choice. This was true for both *Oleria* subspecies, demonstrating that female and larval choices were highly correlated. Also, larval performance has showed positive by correlated with these preferences. Larval development time did not differ significantly between the two host plants. Pupal development was prolonged when larvae fed on *S. anceps*. Significantly larger pupal length and higher larval survival rate both showed that *S. mite* is a better host than *S. anceps* for both subspecies. The widths of the pupae were not taken into account as they revealed only the water content of the pupae. The differences in length are probably due to the fact that larvae eat less on *S. anceps*, leaves of which are tougher than those of *S. mite*. Also, *S. anceps* may be less digestible for the larvae than is *S. mite*. To define better the differences in the suitability of the four potential host plants to the larvae, analyses of their chemical compounds would be helpful. Such studies would show if *S. mite* is richer in nutrient, or perhaps possesses chemicals, which are more attractive for both larvae and females.

Relationship between biology, performance and predation

We discussed in the first part of this chapter that larval development was slightly longer for *O. o.* ssp. than for *O. o. agarista*. This fact is in conflict with the notion that larval development should be as short as possible at those sites were predation appears to be comparatively high. In this part we will discuss how larval activity is related to larval survival.

We first compared the three main activities, resting, eating, and walking, between the two subspecies at different times of day. Thereafter, eating and walking were grouped together as activities during which larvae are more exposed to natural enemies in the field. The comparison showed that *O. o.* ssp. spent more time resting than did *O. o. agarista.* This mean to stay more hidden. than the other. Both subspecies tended to rest more in the morning from 7.00 hours till noon than at other times of day. *O. o.* ssp. fed during the afternoon hours whereas *O. o. agarista* (= Km30) fed mostly at midday. The main activities of *O. o. agarista* during the afternoon were feeding and walking. The observation that *O. o. agarista* larvae move more than these of *O. o.* ssp. can be related firstly to the fact that this subspecies suffers less predation than the other (Machado & Freitas, 2001) and secondly to the observation that *S. mite* is less abundant on the NE slope. This implies that larvae would need to wander around more to find their less abundant host plants. *O. o.* ssp. on the other hand is less active: it rests in the morning and feeds from midday on. Larvae usually rest on the underside of the host plant leaves, at the base of the stem, or on other objects near the host trunk.

The field experiment in which eggs were placed either on the host plant or on objects next to the plant showed that on the SW slope eggs had a higher rate of survival when placed on objects near the host plant rather on the plant. On this slope, when plants were protected by glue, egg disappearance was significantly higher on non-protected plant. This observation and the fact that O. o. ssp. larvae are less mobile than those of O. o. agarista led us to the conclusion that this subspecies could be more exposed to natural enemies and thus requires protection of eggs from predators that walk on the leaves. Also, the larvae seem to avoid being exposed on the leaves for too long and therefore reduce the risk of possible predation. This fact will be discussed here in terms of predation and not of natural enemies, as parasitism was never observed in the experiments. As these behaviours were observed only on the SW slope, they are probably due to one or several predator groups that climb on the host plants and do not occur on the NE side. The one predator group that is abundant, climbs on the plants, and patrols their leaves, are certain ant species. However, the utilisation of Barber traps in ant capture

may be discussed as the quantity of the different genera of ants captured may vary drastically depending on ethological and physiological factors of the insects (Seifert, 1990). When manually collected in the field, the only abundant predatory genus that was exclusively found on the SW side but not on the NE side was the Ponerinea genus Ectatomma. Ectatomma are medium-sized ants (~ 1 cm.) which showed careful observation behaviour of the leaves and after 5 to 10 min of exploration started grooming. Ants of the genus Ectatomma may be categorised as "calm" ants in comparison to ants of genera such as Pheidole and Camponotus, which were found to show very nervous activity when manipulated. In other genera observed, grooming was the first activity done by the ant after being laid on the paper dish that sealed the pot of the plant, as for example Camponotus, Pheidole and Crematogaster usually first run. The aptitude to climb on the plant and to search food on them is more easily assessed for “calm” ants than for “hectic” ones, as in this latter case the fact of climbing may be completely aleatory.

Relationship between ants and butterflies has been studied mostly in terms of predation, However cases on mutualism or associations has been reported. Previous works have revealed different types of antagonistic relationships between ants and herbivores: ants may prey on immature satges of butterfly (Letourneau, 1983; Heads & Lawton, 1985; Costa et al., 1992; Freitas & Oliveira, 1996) herbivore eggs may be removed by ants and dropped to the ground, resulting in enhanced egg mortality (Letourneau, 1983), or plants may be occupied by ants, which can affect the oviposition behaviour of the butterflies (Inouye & Taylor, 1979; Freitas & Oliveira, 1996). The presence of ants on plants is often due to the production of nectar in extrafloral nectaries (EFN's) by the plant (Turlings & Wäckers, 2002). On the other hand, obligatory mutualism has been found in many Lycaenid species (Atsatt, 1981a, b; Pierce & Edgar, 1985) for which ants are necessary for larval development. Cases of facultative mutualism was reported in riodinids where ants defend the butterfly larva on their host plant against predators (DeVries, 1991). An intermediate case between predation and mutualism was found in an *Ectatomma* ant species and the Ithomiinae genus *Mechanitis* on a *Solanum* species. The ants profit from the damage done by the young larvae on the leaves by consuming the exsudates (Young, 1978c). In this special case, neither predation by the ants on the larvae was noted nor did the ants offer protection against predators. Published studies of *Ectatomma* have mostly been related with their habit of feeding on EFN's (Costa et al., 1992; Morrone et al., 2000; Bluethgen et al., 2000; Apple & Feener, 2001), and they are considered as potential predators. However no special predation events were

recorded by this genus on herbivores as it is the case with other genus like *Myrmica, Formica* (Heads & Lawton, 1985) *Camponotus* (Oliveira et al., 1987; Machado & Freitas, 2001) and *Pheidole* (Letourneau, 1983; Machado & Freitas, 2001). Even so, studies of *Ectatomma ruidum* have revealed that immature stage lepidoptera may represent up to 5.4% of their alimentation (Ibarra-Núñez et al., 2001). We cannot assert that *Ectatomma* are the ultimate cause of *O. o.* ssp. laying eggs next to the host plant, as no predation act was observed when eggs were offered to the ants. However, they are considered as predators in the literature (Schatz & Wcislo, 1999; Schatz et al. 1999) Furthermore, we observed that *Ectatomma* individuals usually spend most of their time on plants (*Solanum spp.* and others). And importantly, they are present and common only on the SW side. Therefore, we suggest that they may constitute some of selection pressure, either as egg predators, or in repelling ovipositing females leading to the behaviour laying eggs close to the host plant rather than on the host plant itself.

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Chapter 3

Genetic (RAPD) diversity within and between Oleria onega agarista and Oleria onega ssp. (Ithomiinae, Nymphalidae, Lepidoptera) in north-eastern Peru

Based on:

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Genetic (RAPD) diversity within and between Oleria onega agarista and Oleria onega ssp. (Ithomiinae, Nymphalidae, Lepidoptera) in north-eastern Peru. In preparation for submission to Molecular Ecology

INTRODUCTION

It is widely accepted that distributions of species arise as a product of three processes: speciation, extinction and transformation (e.g. Gaston, 1998). As a general picture, species having a larger distribution range, have also a greater probability to be divided by a barrier (climatic, geographic, environmental or other), than do species having a small range, and are thus more susceptible to speciation processes. The frontier where two partially interfertiles population or species meet (e.g. two different mimicry forms of unpalatable prey taxa), is known as a "hybrid zone". Fundamentally, a hybrid zone is a cline or set of clines between two parapatric or sympatric hybridising taxa (Hewitt, 1988), and they are usually very stable, while contact zones are sites were two species meet without hybridisation. Because of the strong selection on rare forms, known as frequency dependent selection, hybrid and contact zones are generally narrow (5-10 km) (Barton & Hewitt, 1985 1989; Mallet, 1989; Jiggins et al., 1996). Hybrid zones are maintained by two antagonistic processes: on one hand selection (Mallet & Barton 1989), ecological factors and adaptations (Jiggins et al., 1996) which act against gene flow, and on the other hand by dispersal that tend to lead to speciation.

In this study, we clarify the taxonomical status of two Ithomiinae subspecies and their morphological hybrids. This study concentrated on two Ithomiinae subspecies: *Oleria onega* ssp. nov, thus far undescribed (Lamas, pers. comm.), and *O. o. agarista* (C. Felder and R. Felder, 1862). They inhabit the easternmost mountain chain of the Andes before the Amazonian plain, in north-eastern Peru, near the small town of Tarapoto. This mountain chain, called the Cerro Escalera, is considered an ecological barrier for various organisms, principally butterflies (Joron 2000, Joron et al. 2001; Shulte 1999; Mallet, 1989, 1993). On the Tarapoto side, where the climate is hotter, we found *Oleria onega* ssp., while only *O. o. agarista*, the subspecies of the lowland forests, was found on the other slope characterised by a wetter and cooler climate. The two subspecies cohabit in only two locations: Estero, near Shapaja, and the Bocatoma of Ahuashiyacu, near Tarapoto (Chapter 1). In this latter site, both subspecies are present but apparently do not hybridise, whereas in Estero morphological hybrids were observed. Therefore, Ahuashiyacu could be considered as a "contact zone", while Shapaja could be an "hybrid zone".

Oleria onega agarista (C. Felder and R. Felder, 1862).and *Oleria onega* ssp. are considered as conspecific by Lamas (Lamas pers. comm.). However, the status of *O. o.* ssp., as a subspecies or a

completely different *Oleria* species, is not already established. *O.o.* ssp. differ morphologically from *O. o. agarista* by the narrower black edge of the wings, and by two black bands on the hindwing that are never connected (Lamas, pers. comm.), whereas in *Oleria onega agarista* a transversal band connect these two bands on their middle part on the Cu1 and Cu2 veins. The morphological hybrids are recognisable by an incomplete or absent transversal band. As hybrids are rare in the zone where they sympatric, we can suppose that a biological barrier exists, preventing crossings. Therefore, in a previous study (Chapter 1), the two subspecies were put together to interbreed in laboratory, in a search for eventual pre- or post zygotic barriers. However, females born in cages never accepted males, regardless of whether they were from the same subspecies or not, and regardless of whether males were wild or born in cages. Some behavioural differences between *O. o. ssp.* and *O. o. agarista* were also observed. Indeed, females of the first species lay eggs up to 1 meter away from the host plant, whereas *O. o*. *agarista* lay them mostly on the leaves of the host-plant (Chapter 1).

To gain a better understanding of the genetic relationship between these two subspecies, it was important to assess the range of their genetic variation. A suitable tool is the RAPD technique (Williams et al, 1990) which provides a virtually unlimited number of neutral DNA markers (Williams et al, 1990) and is therefore an appropriate method for initial, overall analysis of variation between populations. In addition, material for RAPD can be collected in the field and stored in alcohol, avoiding the necessity for freezing or immediate processing, required for other techniques like isozymes (Bartish et al. 1999). Moreover, RAPD markers can easily detect differences among populations and species of different organisms, including plants (Ayres et al., 1999; Bussell 1999, Bartish et al., 1999; Skotnicki et al., 1999; Comes & Abbott, 2000; Guadagnuolo et al., 2001a, 2001b), invertebrates (Ritchie et al., 2001; Moya et al., 2001) and vertebrates (Clausing et al., 2000; Vucetich et al., 2001). Two major and often mentioned drawbacks of RAPDs markers are the lack of reproducibility and the loss of complete genotypic information, due to the fact that most RAPD bands are dominantly inherited. However, the problem of non-reproducible fragments can be highly reduced by using only high-quality DNA and by careful optimisation of the PCR conditions (Guadagnuolo et al. 2001c, Wiesing et al.1994). Moreover, an Analysis of Molecular Variance (AMOVA), which is not influenced by the dominance of the used markers, can be used to determine the partitioning of RAPD variation between and within populations (Huff et al. 1993).

The aims of this study were: i.) to elucidate, at least partly, the taxonomical relationships between the two subspecies by investigating the genetic similarity between them, ii.) to investigate the molecular variation among populations and iii) to determine the status of the hybrids in relation to *O. o.* ssp. and *O. o. agarista*.

MATERIALS AND METHODS

Population sampled:

A total of 120 samples of *Oleria onega* ssp., *O. o. agarista* and putative hybrids between them were collected from 7 populations near Tarapoto and on the Cerro Escalera (Figure 1 Chapter 1) (Table 1). In order to evaluate genetic distances between the two subspecies, we also collected seven specimens of a different *Oleria* species, *Oleria gunilla serdolis* (Haensch, 1909), and five specimens of a different *Ithomiinae* genus, *Hyposcada zarepha flexibilis* (Haensch, 1909).

Three different conservation methods were tested: (i) in a subset of samples, butterflies were dried and conserved in well closed boxes with silicagel, (ii) for a second subset the butterflies were conserved in 70% alcohol, and (iii) 99% alcohol was used for the third subset.

Table 1 : Sampling localities of *Oleria onega ssp., O. o. agarista, O. gunilla* serdolis, and *Hyposcada zarepha* flexibilis, number of individuals analysed and abbreviations used in the results of the cluster analysis presentedd.

Populations	Nb of individuals	Abbreviation
Oleria onega ssp		
Shilcayo	15	S
Urahuasha	7	U
Ahuashiyacu	11	A
San Roque	13	SR
Shapaja	9	SH
Oleria onega agarista		
Km30	18	K
Km28	5	KM
Ahuashiyacu	2	AA
O. o. ssp*/agarista Hybrids*		
Shapaja	7	HSH
Oleria gunilla serdolis (SanRoque)	6	SRogs
Hyposcada zarepha flexibilis (S. Roque)	2	SHhz
*Hyposcada zarepha flexibilis (*Shapaja)	1	SRhz

DNA extraction

Samples conserved in alcohol were washed three times with deionized water, and then dried with paper.

In order to obtain enough DNA for numerous RAPD reactions, the DNA extractions were performed on abdomens, which contain a large number of cells and are consequently rich in DNA. However, the ends of abdomens were reminded to avoid possible contamination by the spermatophore of male origin in females, as well as the sclerified parts of male abdomens (Aubert et al., 1999; Schneider et al., 1997). The abdomens were ground in 2 ml Eppendorf tubes containing liquid nitrogen. Extractions were then performed using the QIAGEN DNeasy Kit for animal cells (QIAGEN Inc.) according to the manufacturer's instructions and DNA was resuspended in TE (pH 8, Tris 10mM-EDTA 1mM). Because of degradation of the dried material, DNA extractions were finally performed only on the 133 butterflies conserved in alcohol. The integrity of DNA was tested on a 0.8% agarose gel, and only 96 out of the 133 extracted individuals were suitable for subsequent RAPD analyses. DNA was

quantified using a WPA lightwave S2000 spectrophotometer and samples were then diluted at a concentration of 5 ng/µl and stored at -20°C.

RAPD markers

All the PCR reactions were carried out in a final volume of 25 µl containing 25 ng genomic DNA, 2.5 µl10x PCR buffer (with 1.5 mM $MgCl_2$), 0.25 µL 10mM dNtp, 0.5 µl primers (10 pmol/µl), and 0.1 µl Qiagen Taq polymerase (QIAGEN Inc). Amplifications were performed with a Biometra T-Gradient thermocycler with the following profile; 3 min at 94°C, followed by 39 cycles at 94°C for 45 s, 40°C for 45 s, 72°C for 1 min. and a final extension step of 10 min at 72°C . PCR products were mixed with 1/5 vol. loading buffer and separated on a 1.2 % (w/v) agarose gel, containing 0.4 µg/ml ethidium bromide, in 0.5 X TBE at 100 V for 1 hour. DNA fragments were then visualised under UV light.

We tested 23 random primers of the series OPB, OPP and OPT (Operon Technologies, Alameda California) on seven samples of the two subspecies, and repeated amplifications three times. Negative controls were added at each PCR. Seven of these primers, OPB-01, OPB-11, OPB-12, OPB-15, OPT-01, OPT-05, OPP-04, gave clear and reproducible results and were thus used on all samples, whereas OPB04, OPT04, OPP06 were not taken in account because of differences between replicates were too great. OPB-02, OPB-05, OPB-06, OPB-13, OPB-14, OPB-16, OPT-02, OPT-03, OPP-01, OPP-02, OPP-03, OPP-05 were tested but did not amplify and OPB-03 showed only one monomorphic band. The results for this primer were thus discarded.

Data analysis

RAPD markers were scored in a binary form as presence or absence of amplified bands (respectively 1 and 0) for each sample.

Cluster analysis

We performed cluster analyses using the CLUSTER package (http://www.biology.ualberta.ca/jbrzusto) to determine if samples of the same species formed groups according to their morphological appearance. The asymmetrical Jaccard's coefficient, which considered

only shared presence, was used to calculate similarity between samples and to generate a similarity matrix. This latter was then used to produce an UPGMA (Unweighted Pair-Group with Arithmetic averaging) dendrogram, visualised with TREEVIEW (Page 1996)

Principal Coordinates Analysis

The similarity matrix calculated with Jaccard's similarity coefficient between RAPD's samples was converted into a distance matrix (D=1-S). The distance matrix was used to perform Principal Coordinates Analysis (PCoA) using the R4 (Beta version) package (P. Casgrain & P. Legendre, Université de Montréal). Results were graphically represented in a bivariate Scattergram using StatView (SAS institute, Inc.)

In order to test the statistical significance of groups determined by both cluster and PCoA analyses, a Mantel test was performed (999 permutations) using the R4 (Beta version) package. The similarity matrix obtained by genetic data (excluding the outgroup species *O. gunilla serdolis* and *H. zarepha flexibilis*), and a generated matrix in which a distance value of 1 was assigned between two samples of a same subspecies and a value of 0 was assigned between two samples of different subspecies, were converted into a distance matrix and compared pairwise.

Analysis of Molecular Variance (AMOVA)

Analysis of Molecular Variance (AMOVA; Excoffier et al, 1992) was performed using the software ARLEQUIN 1.1 (Schneider et al 1997) in order to describe the genetic variability among and within subspecies and populations. Only the data for the two *Oleria onega* subspecies were considered in these analyses, avoiding hybrids and the outgroup. One population of O. o. *agarista*, that of Ahuashiyacu, represented by fewer than five individuals was not taken into account.

RESULTS

RAPD markers

A total of 92 fragments were scored, the size of which ranged between 300 bp and 1500 bp. All the fragments were polymorphic among individuals.

Four of the total of 92 fragments were present only in the outgroup species. Thus, out of the remaining 88 fragment present in the *Oleria onega* complex only, 57 were present in both subspecies, 76 were present in *O. o.* ssp and 69 in *O. o. agarista.* Out of the 57 fragments common to *O. o.* ssp and *O. o. agarista*, 30 were present in the hybrids. Five fragments specific to *O. o.* ssp. and only two specific to *O. o. agarista* were found in hybrids. In some cases, fragments that were scarce in the subspecies, were frequent in the hybrids, but no fragment was specific to hybrids.

The primers used varied widely in their ability to detect variation between and within populations. Indeed, the mean number of polymorphic fragments among populations, scored individually for each primers varied between 1.89(OPP-04) and 8.55 (OPT-01) (Table 2)

Table 2 : Characteristics of fragment variation generated by seven oligonucleotide primers in the RAPD analysis of nine Oleria onega ssp. populations.

Operon primer code	Total fragment polymorphic	Mean no. of polymorphic fragments per population
OPB01	10	02.89
OPB11	12	06.22
OPB12	14	04.44
OPB15	14	06.89
OPT01	18	08.55
OPT05	17	08.44
OPP04	6	01.89
Mean	11.44	
Total of polymorphic fragment	91	

Data analysis

Cluster analysis based on the RAPD-generated similarity matrix separated the individuals into five main groups (Figure 1). Group I is constituted by the different populations of *O. o* ssp. However, while most of the individuals of the populations of San Roque (SR), Shapaja (Sh) and Shilcayo (S) are gathered in this group, those from Ahuashiyacu and most of those from Urahuasha show a higher level of within-population genetic diversity and are indeed dispersed throughout the dendrogram. Group II is composed of the *O. o. agarista* individuals from population Km30 (K) and the two individuals of *O. o. agarista* of the Ahuashiyacu mixed population (A49, A51). Hybrids from Shapaja (HSH) and the *O. o. agarista* individuals from the Km28 (KM) population, as well as some individuals of *O. o.* ssp. from Urahuasha (U76-U78) are clustered together in Group III. Finally, the groups IV and V include, correspond to the two species used as "outgroup", *Oleria gunilla serdolis* (SRogs) and *Hyposcada zarepha* (SRhz and SHhz).

The results obtained by the Principal Coordinates Analysis showed that the first three principal factors accounted for 12%, 10%, and 7% respectively, of the total variation (Figure 2). Despite these relatively low values, the two *Oleria onega* subspecies and their hybrids are clearly separated (Figure 2). However, as was observed with the cluster analysis, some of the individuals of each subspecies are not grouped according to their morphological appearance. Here again, hybrids are intermixed with the *O. o. agarista* individuals of population Km28, which are geographically the most distant one population Shapaja.

A weak but significant correlation was observed between the genetic data and the morphological appearance of the *Oleria onega* individuals. Indeed, the Mantel correlation value between the RAPDs based distance matrix and the generated matrix (where a distance value 1 was assigned between individuals of a different subspecies and 0 between individuals of the same subspecies) was $r = 0.14$ ($p = 0.001$).

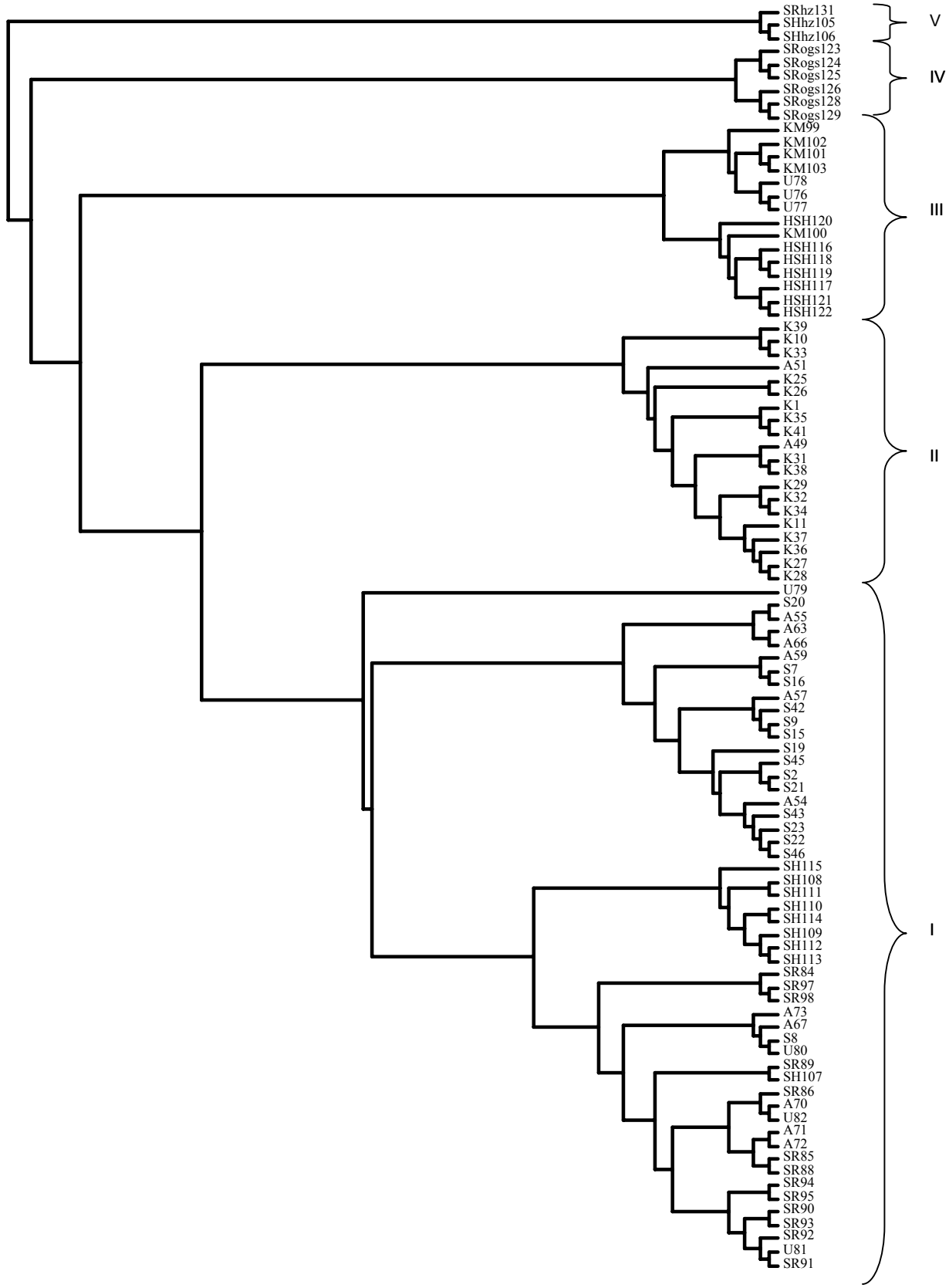

Figure 1 UPGMA dendrogram based on Jaccard similarity coefficient for *Oleria onega* ssp., *O. o. agarista, O. gunilla serdolis*, and *Hyposcada zarepha flexibilis*. Group I is consituted by *O. o.* ssp., group II by *O. o. agarista*, group III by hybrids and one *O. o. agarista* population, group IV by *O. gunilla serdolis* and group V by *Hyposcada zarepha flexibilis*.

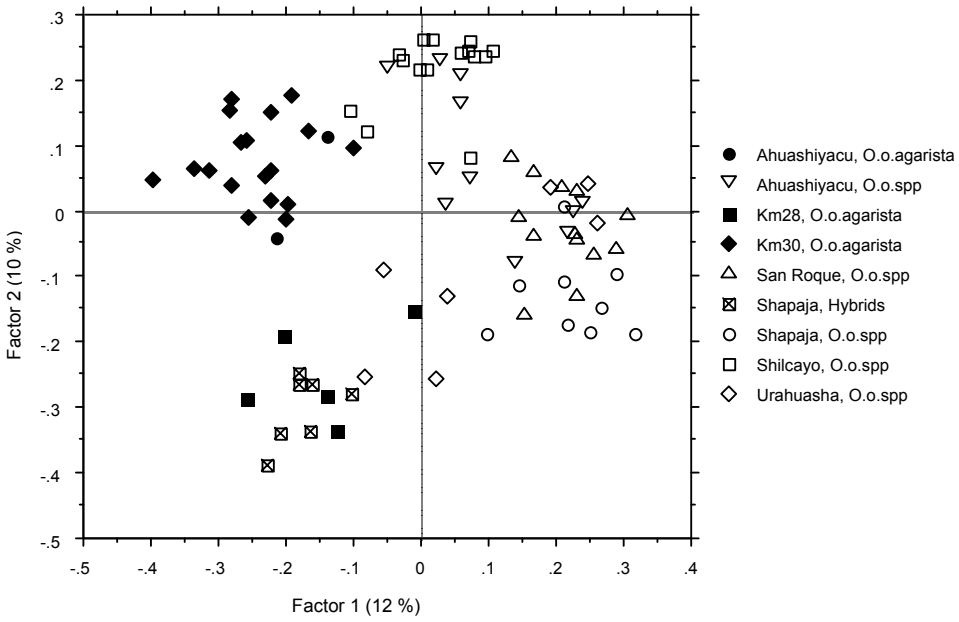

Figure 2 : Principal components analysis of 87 *Oleria onega* butterflies based on a squared Jaccard distance matrix on the total (92) RAPD markers.

AMOVA analysis

Partitioning of molecular variance was calculated among subspecies and among populations (Table 3). When using subspecies as a variable group, 22.26% of the total variability was attributable to differences among subspecies and 77.74% to differences within subspecies. Grouping the material according to *Oleria onega* geographical populations, only 13.22% of the variability occurred between subspecies, 19.59% among populations within subspecies, and 67.20% within subspecies. The pvalues for all analyses of variance were highly significant (Table 3).

Table 3 : AMOVA for RAPD phenotypes in *Oleria onega agarista* and *O. o.* ssp. The total dataset contains 80 individuals from 7 populations (5 *O. o.* ssp. and 2 *O. o. agarista*), using 92 RAPD markers. Two analyses were conducted: the first among and within the two subspecies and their hybrids, and the second among subspecies and their populations and within populations. Percentile distribution of the variance components, as well as P value and fixation indices are given. F statitstics are defined by three fixation indices : Fct=proportion of differentiation between subspecies, Fsc=differentiation among populations within subspecies, Fst=the global differentiation of populations.

Source of Variation	d.f.	Variance component	% total variance	p-value	Fixation indices
Among subspecies	2	2.59 Va	22.26	p<0.001	F_{ST} 0.2226
Within subspecies	84	9.04 Vb	77.74	p<0.001	
Among subspecies	1	1.58 Va	13.22	p<0.001	
Among populations Within subspecies	2	2.25 Vb	19.59	p<0.001	F_{SC} 0.2257 F_{ST} 0.3280 F_{CT} 0.1321
Within populations	71	7.71 Vc	67.20	p<0.001	

Significance test (1023 permutations)

DISCUSSION

Genetic Markers

The large set of markers obtained in this study confirmed the ability of the RAPD technique to differentiate organisms at the genetic level. We detected a high level of polymorphism between the two studied subspecies, but also within each subspecies. Indeed, almost none of the amplified fragments were present in all the individuals. Surprisingly, the high polymorphism of the studied organisms led to difficulty in interpretation of the results. Indeed, only few markers were constantly present within subspecies and even populations. It was thus difficult to assess whether these fragments were specific to a subspecies or simply more frequent in some populations than in others.

Since RAPD markers are known to be highly polymorphic, it would be interesting to use more conserved markers (i.e. isozymes) or to investigate more conserved sequences, in order to better assess the genetic relationships between these butterflies.

Differentiation of the subspecies and hybrids

Both Cluster and PCoA analyses separated *O. o.* ssp and *O. o. agarista* populations into two distinct groups. The relative distance between them, lower than that separating both taxa from *O. gunilla serdolis,* support their classification as two different subspecies (Figure 2). However, the differentiation of the studied individuals in two groups (i.e. subspecies) was not clear, as demonstrated by the low correlation value (Mantel's r = 0.14, P = 0.001) between RAPD markers based matrix and the built distance matrices. This low value can be explained by the high polymorphism detected within the studied subspecies, which partly hides the differences between them, as demonstrated by the AMOVA results. In addition, some of the individuals of *O. o.* ssp. (U76, 77, 78) were grouped close to *O. o. agarista* both by cluster and PCoA analyses and the population Km28 was closer to the hybrids than to the other populations of the same species.

Independently of the type of marker used, one would expect the F1 hybrids between two taxa to have a position intermediate between the two studied subspecies in a genetic analysis (e.g. Guadagnuolo et al. 2001b). In the present study, the morphologically detected hybrids seem to be more closely related to *O. o. agarista* than to *O. o.* ssp (Figure 2). The degree of hybridisation and the probability of backcrossing with either or both parents were impossible to calculate as too few hybrids were collected, and because of the high polymorphism among individuals and populations. Nonetheless, our results suggest that at least some of the "hybrids" could be the result a backrossing between a F1 hybrid and *O. o. agarista* as the recurrent parent.

Hybrids occurrence and viability

In the Ahuashiyacu population where we found both *Oleria onega* subspecies, no morphological hybrids were observed, whereas in Shapaja hybrids are frequent. In addition, the genetic distance between the Ahuashiyacu *O. o. ssp* and *O. o. agarista* individuals is lower than that between Shapaja *O. o.* ssp and hybrid individuals. It is difficult to speculate on the reasons for these results, but they

allow us to formulate several hypotheses. The first is that a reproductive barrier exist between the two subspecies in the population of Ahuashiyacu, that could lead to a genetic isolation. Unfortunately, it was impossible to determine whether such a barrier reducing hybridisation exists. Indeed, neither the two subspecies nor the hybrids ever reproduced in captivity, and it was thus impossible to perform crosses in laboratory. Nevertheless, natural morphological hybrids from Shapaja did not show reduced viability, and produced fertilised eggs with normal development. These observations suggest that genetic incompatibility between *O. o.* ssp. and *O. o. agarista,* if it exists, is only partial. A second hypothesis is that barriers to gene flow are not just associated with a few strongly selected colour pattern loci, but dispersed across the genome as found on the sister group Heliconiinae (Jiggins et al. 1997), and are a result of divergence in mate preferences, warning colour and ecology without hybrid inviability or sterility (Jiggins et al., 1996 McMillan et al, 1997). Even so, a decisive explanation for the occurrence of hybridisation in one zone of sympatry and not in the other, is hard to give.

Differentiation and relatedness between populations

Because of the recent modification of the environment by extensive deforestation, with the consequent fragmentation of natural habitats, the probability of current gene flow between populations is low. The Cerro Escalera represents an additional physical barrier, which is thought to completely separate the populations of Estero from those of Tarapoto. However, our results suggest, that gene exchange between populations, as well as between *Oleria onega* subspecies, has occurred at least in the past. The results of the AMOVA, showing a higher genetic variation within populations rather than between them, is a pattern frequently observed in both plants and animals (Skotnicki et al., 1999; Comes & Abbott, 2000; Moya et al., 2001). In the present case, this is an additional indicator of a high rate of gene flow. The high percentage of polymorphic markers that was observed within subspecies, becomes drastically lower at the population level (Table 3). In reality, this is due to the fact that the majority of fragments were absent in one or other population but considered as present at the subspecies level. Only a low percentage (max. 15 %) of monomorphic fragments was observed within a population.

The similarity of some *O. o.* ssp individuals of Urahuasha (U76, 77, 78) with the *O. o. agarista* individuals of the Km28 population is difficult to explain, since Urahuasha is the upper population on the SW slope (fig 1, p. 19) and is separated from KM28 by the highest peaks of the Cerro Escalera.

Moreover, only *O. o.* ssp. individuals were found in Urahuasha and only *O. o. agarista* at Km28. The possibility of a recent contact between these populations is thus extremely unlikely. Moreover, the other Urahuasha individuals are mixed with the Shapaja and San Roque populations, which are the geographically most distant ones. The most probable explanation we can offer for this fact, is that the Urahuasha population has been in contact with *O. o. agarista* in the past, and that these three individuals may be a results of hybridisation and subsequent back-crosses between F1 hybrids and *O. o.* ssp. as the recurrent parents.

Another unexpected result is the clear separation between the two *O. o. agarista* populations of the NE slope of the Cerro Escalera (Km30 and Km28), by both cluster and PcoA results. Moreover, the Km28 population was always grouped with the hybrids, while the two *O. o. agarista* individuals from the SW slope (Ahuashiyacu) were grouped with the Km30 population.

Since the Km28 population of *O. o. agarista* is not only genetically close to the analysed hybrids but also relatively well separated from the other populations of the same species, it is conceivable that these are the results of past hybridisation events between the two studied subspecies. Subsequent isolation from *O. o.* ssp. in Km28, and from *O. o. agarista* in Urahuasha could have led to a convergent evolution. That would mean that both subspecies are under the effect of a speciation process, which could lead to their extinction through hybridisation, as already shown by several studies on both plant and animals (Huxel, 1999; Wolf et al. 2001).

It should also be noted that the hybrids from Shapaja, are more closely related to the three *O. o.* ssp. individuals from Urahuasha cited above (U76, 77, 78) than to those from Shapaja. Morphologically (data not shown), hybrids have more characters in common with *O. o. agarista* than with *O. o.* ssp. However, we found them to share five RAPD characters in common with *O. o.* ssp. versus two with *O. o. agarista*. This may suggest that the morphological characters of *O. o. agarista* are dominant over those of *O. o.* ssp., but examination of a larger number of hybrids may be required to make this assessment, as well as studies of genes coding for the morphology. On the other hand, hybridisation may lead to the introgression of only neutral alleles, leaving intact parts of the genome that are under selection and define species identity (Buerkle et al., 2000); this is translated by organisms that show hybrid ancestry but retain the pure phenotype. Moreover, because of selection against recombinant phenotypes, morphological measurement may consistently underestimate the true proportion of hybrids in true phenotypes (Rieseberg et al., 1999a, 1999b)

Another important observation is that, during the three years of field work, the number of *O. o. agarista* individuals of Shapaja and Ahuashiyacu, were decreasing rapidly (data not shown). Therefore, at least in Shapaja, a selective pressure seems to act against this species, while the hybrids possess some ecological or physiological advantages that allow them to persist in their environment. In another study, it has been shown that extinction process may occur in five or fewer generations (Wolf et al., 2001), a result that fits with our study. In order to test this hypothesis, it would be interesting to genetically resample the Ahuashiyacu population, where no morphological hybrids were found, after several generations. Nevertheless, the similarity of all the *O. o. agarista* individuals of the Km28 population with the morphological hybrids could be an additional indication that support this hypothesis, since hybrid phenotype in Km28 may have disappeared even though gene information is still present. As discussed above, this population could be the result of past hybridisation events, and where selective pressure has acted in favour of the *O. o. agarista* form. From our results, we can postulate that populations near the top of the mountain may have been in contact in the past, but that selective pressures led them to adapt their morphological features to the pattern corresponding to the area: that of *O. o. agarista* on the NE slope of the mountain and that of *O. o.* ssp. on the SW slope.

CONCLUSION

The objective of this study was to analyse genetic variation among two *Oleria onega* subspecies and their relative hybrids. We tried also to understand the speciation process and variation among populations in relation to their geographical situation. The most pertinent finding of this study was that the two subspecies are distinct, even though polymorphism among population is high within each subspecies, and that they are well distinct from the outgroup. Variation is greater within populations than among them, nevertheless we believe that gene flow between them has occurred in the past (and still occur when allowed by topography and climate). Hybrids are distinct from both subspecies, but the population near the top of the mountain still shows traits of probable hybridisation events in the past. On both slopes, selective pressures had led the rare forms to evolve toward the more common one, and the only population where hybrids seem to suffer less evolution contraints is Estero, near Shapaja.

In this study, the choice of RAPD markers were helpful in detecting the differences between subspecies, in identifying hybrids and indescribing variation within and among populations. RAPD amplification provided a large set of polymorphic markers. This is not astonishing as this technique can detect variation in both coding and non-coding sequences, and the length of the primers allows the amplification of a large number of fragments with a single primer. Nevertheless, further studies with other markers are required to confirm the taxonomical statute of each subspecies, and to measure the extend of hybridisation between them. It would be interesting to study the loci coding for the colour-pattern differences and determine whether one of the patterns is dominant over the other.

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Chapter 4

Modelling of the Distribution of *Oleria onega agarista* and *Oleria onega* ssp. (Ithomiinae, Lepidoptera), in Peru Using Geographic Information Systems and Geospatial Analysis

Based on:

Gallusser, S.; Bouzelboudjen, M.; Rahier, M.

Modelling of the Distribution of Oleria onega agarista and Oleria onega ssp. (Ithomiinae, Lepidoptera), in Peru Using Geographic Information Systems and Geospatial Analysis.

Unpublished chapter

INTRODUCTION

The Oleria onega subspecies complex

Oleria onega is a complex of ithomiinea butterfly subspecies (Lamas, pers comm.) that extends to all the south-American tropical forest and counts some fifteen subspecies, some of them are yet undescribed. Two of these subspecies are found near Tarapoto, in north eastern Peru: *O. o. agarista* (C. Felder and R. Felder) and *O .o.* ssp. a recently discovered subspecies. Their taxonomical status is not completely defined, between subspecies or two totally different subspecies (G. Lamas, pers. com.). Their genetical variations (Chapter 3) revealed that gene flow had occurred between populations (and may still occur when allowed by topography and landuse). These two subspecies are geographically separated by a mountain chain, the "Cerro Ecalera" which constitutes a big biological barrier, with a quite different fauna and flora on each side (Mallet, 1989, 1993; Shulte, 1999; Joron 2000, 2001). *O. o.* ssp. lives mostly on the southwestern (SW) slope, whereas *O. o.* agarista extend his range on the northeastern (NE) slope and all the Huallaga Valley till Yurimaguas. Four Solanaceae species are used by Oleria onega as host plants (Mallet pers. comm.) : *Solanum mite* (Ruiz & Pav.), *Solanum anceps* (Ruiz & Pav.), *Solanum angustialatum* (Bitter), and *Solanum uleanum* (Bitter) (Knapp 1997). On the NE slope, three of them are found together: *S. mite, S. anceps*, and *S. uleanum*; whereas on the SW slope only *S. mite* was found. *S. angustialatum* grows on the upper part of the mountain.

Geographical information systems and their recent applications in biology

In the strictest sense, a GIS is a computer system capable of assembling, storing, manipulating, and displaying geographically referenced information, i.e. data identified according to their locations. GIS is not only a cartographic computation system, but also an analytical tool that allows storage, manipulation and analysis of data. GIS makes the link between discrete and continues variables by a complex tissue of spatial relations on the same map, and allows treating quantitative and qualitative data. Several applications have been used in environmental sciences. For example, the soil structure and functioning (Cosandey et al., 2002) have been studied in 2D and 3D (Mendonca et al., 2000).

The application of GIS to biological sciences increased in the last decade with a focus on habitat and species distribution on different organisms indeed birds (Maurer, 1994), plants of the Rocky Mountains (Morain et al, 1993), and aquatic plants (Lehmann, et al, 1994). The different applications of GIS in studies of animal communities were widely described by Mc Laren and Braun (1993). Epidemiological applications were also studied with Lyme disease (Nicholson et al., 1996). In entomology, GIS has been used for study of population structure and geographical variation, using a combination of pure GIS and other spatial geostatistic programs such as Surfer and Isatis (Cesaroni et al, 1997; Kidd and Ritchie, 2000; Margraf, unpublished). A parallel between geographical and genetical (RAPD's) variation was recently demonstrated in Orthoptera (Kidd and Ritchie, 2000; Ritchie et al, 2001).

Using GIS to examine the Oleria onega subspecies complex

The two sides of the Cerro Escalera are climatically different: the NE slope, benefits from a cooler and wetter climate due to clouds coming from the Atlantic that are retained by the mountain, whereas the SW is hotter and dryer. The hotter climate on the SW slope is accentuated by high deforestation because of the proximity of the city, and the resulting open areas constitute a factor restricting the range of the *Oleria* populations. Study of molecular variation revealed that populations are well distinct, but that gene flow may occurs between them (Chapter 3), due to possible contacts in the past, before the increasing deforestation. Even though the Cerro Escalera is a topographical barrier, *Oleria onega agarista* probably found a dispersal path around the mountain because we observed two mixed populations: Ahuashiyacu where the two subspecies co-occur, and Shapaja (not represented in this study) where they hybridise.

Data on distribution of butterflies and their host plants, as well as environmental data, for factors such as temperature, humidity, topography and landuse, were analysed In twenty-three sites. The presence and absence of butterflies, and of the different host plant species, the type of soil, the dimension of the trunk and the density of the understorey were noted. Thereafter, temperature, humidity and the butterfly density were measured at least two times per month. Through statistical and interpolation methods, both the spatial distribution of *Oleria onega* subspecies and environmental data were calculated by kriging and conditional simulations. Thirty other sites were introduced to obtain results concordant with reality, because most of the study area is inaccessible due to topography. For

these synthetic sites no observations on vegetation and soil were available. However, climatic parameters and the butterfly density were estimated based on the data of the most similar study sites. Therefore the interest of this work is in the modelling aspect and the way to use mapping and statistical tools in biology rather than an assertion about actual patterns in the field.

MATERIAL AND METHODS

Ecological database on O. onega ssp*. and O. o. agarista and environmental data:*

The creation of a spatial (Arc/Info®, ArcView®; ESRI, 1996, 1997) and tabular database (Excel; Microsoft, 1997) represented the first stage of work. This database integrated ecological data relevant for the study and is subdivided into two complementary parts; on the one hand, a spatial database incorporating various coverages (a set of thematically associated data considered as a unit, e.g. soils, streams, roads, or land use) (ESRI, 1996). This spatial database contains discrete data (geographic features containing boundaries: point, line or area boundaries) and continuous data (topography, temperature, humidity). On the other hand a descriptive database including data relating to the *Oleria* species (23 stations) was built. Before the implementing of this database, validation procedures adapted for each type of data were defined and applied in a systematic way. To facilitate its data management, a simple structure (tabular files) was adopted for the descriptive database.

The number of males and females observed per day were counted in twenty-three study sites between Tarapoto and the Pongo de Cainarachi, from September to February 1999-2000 and 2000-2001. Data were collected from two to twelve times per month for each site. The coordinates of the sites were established with a Garmin GPS. Each site represents an area of approx. 100x 100 m. As *Oleria* butterflies are active mostly in the morning (Chapter 1 and 2) the number of individuals per day, is in reality the number of individuals observed in the morning (between 8.00h-12.00 h). In each site, the presence or absence and the species of the host plant were noted, as well as soil type, the forest type, and understorey density. Temperature and humidity were measured at least two times per month.

The sites near the road were easily accessible, but not sufficient to allow data interpolation. Therefore 30 synthetic data sites were added, with estimations for mean number of males and females, humidity and temperature, according to the data in the most similar observation site.

Data storage and map construction

A simplified map was drawn from Geographical map No 1658, scale of 1:100,000 (Instituto Nacional Geográfico del Peru). In this simplified map (1cm = 425 m), the two main rivers (Shilcayo, and Progreso river), the road to Yurimaguas (Carretera Marginal), twenty altitudinal contour lines with intervals of 200m, and the limit of the study area were represented as well as the twenty-three observation sites. The limits of areas under different landuse were visually assessed in the field and drawn on the map. All these features were digitalized and corrected using ArcInfo. Lines (rivers, roads, isolines of topography) and polygons (landuse and study area) were generated, as well as a 1x1cm grid including all the study area. Thirty synthetic points were added (from Id 24 to 53, see annex 1), pointing out the chosen place on the View and introducing them as a theme. To each point (observed + synthetic) correspond mean values for number of female and of male, mean relative humidity, and mean temperature degree. The means were done per month, but also the mean for all the different data. The Annex 1 summarize all data used for the geospatial analysis. These represent the mean of observations from September 2000 to February 2001.

Geospatial analysis: geostatistics, kriging, simulations and probabilities

The stochastic approach, based on kriging and multiple conditional simulations, leads to a quantification of uncertainty for both *Oleria* spatial distribution and environmental data.

To calculate the distribution of *Oleria onega* spp. and *Oleria onega agarista* and environmental data (topography, temperature, humidity, etc.) we used a probabilistic approach developed by Matheron [1970]. In fact, direct knowledge of all these parameters in the field can be only partial. The term "regionalized" was proposed by Matheron (1970) to qualify a phenomenon developing in space (and\or in the time) and showing a certain structure. We treated regionalized variables by using the probabilistic theory of the random functions and interprets the regionalized variable as a "realization of the random function" (Chilès & Delfiner 1999). The work of interrogation of the data, then the modelling of their structural properties, establishes under the name of variographic analysis the inescapable phase of any concrete geostatistical study [Chauvet, 1992]. In the probabilistic models, the simplest tool to measure an estimator of quality is the variance. One makes the hypothesis that for a vector h, the increase Z(x + h) - Z(x) has an expected value and a variance independent at the point x, it is the intrinsic hypothesis :

$$E[Z(x+h)-Z(x)]=0 \quad (1)$$

$$Var[Z(x+h)-Z(x)]=2\gamma(h) \quad (2)$$

The function $\gamma(h)$ is called semi-variogram. The semi-variogram is calculated by:

$$\gamma(h)=\frac{1}{2N(h)}\sum_{i=1}^{N(h)}[(z(x_i+h)-z(x_i))^2] \quad (3)$$

z(xi) are the data, xi the points for which the data are available at the same moment in xi and xi + h and N(h) is the number of couples of points distant from h. In any geostatistical study the hypotheses of stationarity and ergodicity should be verified to allow correct estimations (Chauvet, 1992). The Strict Stationarity is a particular case of great pratical importance is when the finite-dimensional distributions are invariant under an arbitrary translation (Chilès&Delfiner (1999). Such Random Functions is called stationarity. Physically this means that the phenomenon is homogeneous in space and, so to speak, repeats itself in the whole space. The sand in the jar is a good image of a stationarity random function in three dimensions, at least if the sand is well sorted (otherwise, if the jar vibrates, the finer grains will eventually seep to the bottom, creating nonstationarity in the vertical dimension). The Ergodicity is an intimidating concept. The practioner has heard that the Random Functions should be ergodic, since "this is what makes statistical inference possible," but is no sure how to check this fact and proceeds anyway, feeling vaguely guilty of having perhaps overlooked something very important. We will attempt here to clarify the issues. In practice, ergodicity is never a problem. When no replication is possible, as with purely spatial phenomenon, we can safety choose an ergodic model. If the phenomenon is repeatable, typically time-dependant fields or simulations, averages are computed over the different realisations, and the only issue (more a physical than a mathematical one) is to make sure that we are not mixing essentially different functions.

Having fitted a model to the experimental variogram , an estimation of our variable can be made at all points of a regular grid by the method of standard kriging (Geovariances, 1998). There are other estimation methods taking into account non-stationarity of the phenomenon (universal kriging, Intrinsic Random Function of order k : IRF-k), (Chauvet, 1992).

Simulations represent a method of estimation which reconstruct the real variability of the variable and allow calculation of probability maps (Chilès and Delfiner, 1999). If Z(x) is the studied regionalised variable, which we consider as a realisation of a IRF-k, and which is known only at n points x, one calls conditional simulation of Z(x) any realisation T(x) of the same IRF-k that passes by the data

at points x. A conditional simulation is thus characterised by the following properties : it has the same generalised covariance as the phenomenon considered, it passes through the experimental points and has a similar distribution (histogram). Although the conditional simulations are not good estimators of the real field (the best estimator being the kriging) they are possible variants of the real field presenting the same degree of variability, respecting what one knows of the real field. They allow to surround the possible fluctuations in the phenomenon (Kimmeier and al., 2001) better than do kriging (which smoothes reality) and the standard deviation of kriging (which gives no indication of the spatial structure of the error of estimation). They remain however connected to kriging : if one builds a great number of conditional simulations, the average of their values at a point will restore the estimated value by kriging, and their variance of corresponding estimation.

The files with the 53 points with their co-ordinates and mean number of females, males, mean relative humidity, and mean temperature (calculated from September to February) were treated with the Isatis software (Geovariances, 1998). An exploratory analysis of the spatial features of the data was done, then the generalised covariance for each variable was calculated, as well as the variogram fitting. Then a cross validation was done for each variable to build the kriging and its standard deviation. From the Cross validation, conditional simulation and probability maps were calculated. Kriging, simulations and probabilities were created on a grid of 0.5 X 0.5 cm.

Interpolation and data grid analyses

Kriging, probabilities and simulations from Isatis were interpolated for the density of females and males, humidity and temperature, with a cell size of 0.5 x0.5 cm, on ArcView. Using the grid-analyst, the maps were extracted from the grid into new maps corresponding to the study area, to study the possible relations between the different variables.

Buffers were created on study sites, rivers and road, using Arcview. For each element, the extension for the buffers was done according to the estimation of their possible interactions with the environment. The buffers of the sites were 200, 400, and 600m, those of the road 100 and 200m and those of the rivers 100, 200 and 300 m. Two buffers were done on the rivers, with the gradient scale of the probabilities to find two males and two females.

Relations between the variables (i.e., topography, humidity, temperature, density of females and males, probabilities of densities of females and males, and buffers on road, rivers and study sites) were assessed visually, and interpreted based to field observation.

RESULTS

Cartography :

In the cartographic part, the twenty-three study sites and the thirty synthetic sites were first represented (fig 1b) on the study area. Five maps were then created with the different numerised themes. On each map, the road, the rivers, the contour line, the sites (only observation sites), the grid, and the limit of the study zone (polygon + line themes) were represented. On these five maps, the distribution of plants and animal communities, and soils information were represented for each study site. As no data were collected on the synthetic sites, they are not represented on these maps.

- presence or absence of the different butterfly subspecies (fig 2a): on the SW slope of the mountain, *O. o.* ssp.. was present in only two sites, and we found a third site (site 7) where the two butterfly subspecies were sympatric. On the NE slope (from the site 15) only *O. o. agarista* was present. These results assess our hypothesis that the Cerro Escalera is a biological barrier for our organisms, even though some dispersal pathways are possible, as demonstrated by the sympatry of the two subspecies in site 7.
- host plant species (fig 2b) : on the SW slope, only *S. mite* was collected, whereas on the NE side *S. mite*, *S. anceps*, and *S. uleanum* were found on the mountain, but *S. anceps* was inexistent in the valley of Progreso. The only host plant that grew on the upper part of the mountain was *S. angustialatum*. Therefore *S. anceps* and *S. angustialatum* seem to be more frequent at high elevation than in the valley. Another pattern that may be related to altitude was noticed with *S. uleanum* and *S. mite*: the plants tend to be dark red with increased pilosity in site 18 (alt 800m) whereas the individuals of the valley were green.
- forest type (fig 2c) : trees with a diameter greater than 70 cm were found in the less deforested zone, mostly on the NE slope, whereas on the SW slope, trees were smaller and cultivated area mare extend more extended. Butterflies were found only in the forested sites.

- understorey abundance (fig 2d) :. in the forested sites, density of the understorey was slightly higher on the NE slope where forest were less disturbed.
- soil type (fig 2e) : the soil was mostly brown (with abundance of clay) with some areas or yellow sand in the valleys. In the forested areas, the percentage of humus was obviously higher. On the mountain, some areas were constituted by white sand and humus, which limited the diversity of plants, butterflies and other organisms. On the upper part, bare rock appears, partially covered by a humus cover up to one meter thick depending on the topography. This suggests that the absence of butterflies in these sites may be due not only the effect of altitude, but also to the soil quality that affect the presence of the host plants and their quality.

On the map of topography (fig 3) we can observe the Cerro Escalera that divides the study area, with slopes of more than 60 degrees (fig 4). The highest peaks reach an altitude of some 1400 m. The SW versant extends to the Cumbaza Valley (Tarapoto) at 300m alt. The NE versant revealed a rougher topography, divided by the small Valley of Progreso which extends to the Amazon Basin.

Landuse shows deforested areas near Tarapoto, and some cultures near Progreso. All the mountainous areas were still forested areas. Some small open areas, not represented on the map, do exist.

Geostatistic: Kriging, Simulations and probabilities maps

Results of the exploratory and variographic analysis of the spatial features of the females, males, humidity and temperature data as well as the cross validations were not represented here.

Kriging and conditional simulations were based on the values of the fifty-three study sites. Kriging was used to study the eventual relations between the density of butterflies, temperature, and humidity, with the topography and landuse.

Simulations were conducted by the method of Turning bands (1000 bands) and were used as graphical representation of the spatial variation of butterfly distributions on the study area. Four simulation maps were done for males and four for females: one based on the dispersion, one on the mean number of individuals, then the largest and the smallest number. The map of the mean of individuals was similar to the kriging map (data not shown). From the simulations, five probability maps were calculated for the males and females according to the mean number of individuals observed per day on an area of ca. 100x100m. This mean number varied between 0 and 5, therefore probabilities

were calculated for 1, 2, 3,4, and 5 individuals (annex 2b). The coloured scale from green to red, revealed the probability (%) of observing this mean number of butterflies on the different points of the grill. For males and females, the probability of observing one individual was high, whereas the probability of observing five individuals was too low to be concordant with reality (data not shown). The sites of highest density were the areas near Shilcayo (site 3) and Km30 (site 18) (the two green areas on the map).

Interpolation and data grid analyse

The data from Isatis were imported to ArcView and visualization was effected on the different variables of the three Isatis results, Kriging, Simulations and Probabilities, and were extracted to correspond to the study area. In order to examine correlation between butterfly distribution and environmental variables, only the kriging and one of the probabilities maps were used and represented here. The probability of finding 2 individuals was the representation that best fit with the reality (fig. 10-11).

The kriging of temperature (fig. 6a and b) revealed that near the top of the mountain and near rivers temperatures were slightly lower (24-26°), and increased to 29° C near the town and in deforested areas. Standard deviations of temperature were low near the sites of measurement, but increased drastically away from the site. Mean temperature predominant in the study area was 26-27° C. In the kriging of humidity (fig. 7a and b), the highest means were in the Km30 area (till 95%), whereas in the Tarapoto area means are situated between 78-83%. The river edge (more obvious with the Progreso river) is clearly represented by a mean of 88-90%. Standard deviations of humidity increased slightly with the distance from the measurement points.

The kriging results of densities of males and females per day were fairly similar (fig. 8a and b, 9a and b) with two high densities sites (km30 and Shilcayo). On the NE slope as well as the Ahuashiyacu area, density was uniform with means between 0.7-1.5 ind. Standard deviations of density of males showed greater uniformity and increased less than those of the females.

The most representative probability was that with two individuals per day (fig 10, 11). The probability of finding two males per day was higher than for females on the NE slope, whereas the highest values of probabilities of finding two females were concentrated in the Km30 site and Shilcayo.

Relationships between butterfly distribution and environmental data

Comparing visually all the kriging and probabilities maps with the topography and landuse grid, and the three buffers, the following relationships are apparent:

- the forested areas show means for temperature between 26-27°C and means for humidity of 85-90%. Forested areas predominate up to 500 m. alt. and deforestation is greater on the SW slope (near the town), then on the NE slope.

- For the kriging of density of both males and females, values between 0.8-1.5 ind./per day were predominant in all the area and these values were found mostly between 85-90% of humidity and 25-27° C. Butterflies were found between 300-800m and in forested areas.

- The probability of finding two ind./day was the closest to reality, and this probability was higher in forested area, mostly on the NE slope. The probability decreased with altitude. Males were easier to find in the lowland than females.

- The highest concentration of butterflies occurred in the Shilcayo and Km30 areas. Both sites are on the mountain flanks but with moderate slopes. On both sites, soil type is similar with clay and with high humus content; on the other sites on the mountain flanks, slopes are more abrupt and rock and white sand predominated.

- According to the four kriging maps, landuse and the topography, a plausible hypothesis for the presence of *O. o. agarista* in Ahuashiyacu, on the SW slope, is that they found a pathway passing at the side limit of mountain, where temperature and humidity are optimal for the butterflies. As no topographic data were available to join the Shilcayo site with the Km30, and as *O. o. agarista* was never found in other parts of the SW slope, this is the only hypothesis we can suggest. This will be discussed further.

- The probability of finding two individuals in relation with the presence of the rivers is higher at the origin of the two rivers (fig 13 and 14). Probabilities were higher near the Progreso river, which flows in areas less deforested than does the Shilcayo river. However we can observe that the proximity of the road reduce the probabilities for the Progreso river (this pattern is more marked for females). Higher temperatures near the road may explain this. No relationships were found between the buffers, the kriging maps and the probability maps.

Distribution of the butterflies is dictated first by food availability. Host plant distribution depends on soil quality, environment (forest) and altitude. Plants were found between 300 and 800m, always in forested areas. A second factor that allows the presence of butterflies is climate (temperature and

humidity). We found that the more suitable parameters for the butterflies were between 25-27° C and 85-90% humidity. These two factors are obviously dependent on the vegetation, and on altitude. In this area, the extend of deforestation depends on topography. The mountains are less deforested because of their steep slopes and poor soil quality. However, temperature and humidity varied greatly between sunny and rainy days. Butterflies were easier to find just after rain, or when the weather was cloudy. In these cases their density increased up to 15 - 20 per morning, whereas during the hottest days, they stay in the vegetation, moving as little as possible (probably early in the morning and at dawn), and are hardly observed. This lack of action during the day explains that the highest probabilities are of finding only two individuals per morning in sites where they are probably quite abundant.

With map-based data there is a strong tendency to ignore or not recognise issues of data quality (Matthews, 1990). Spatial data are subject to the to the same errors and limitations to which any compiled database is subjected. By their very nature, spatial data have an associated spatial accuracy, that is, how close to reality are the shapes and distances depicted on the map. For example, spatial errors can occur because of the resolution of the data. GIS technology allows researchers to combine data from widely disparate scales, projections, or levels of precision. However, one must consider the reliability of the data. For example, a study conducted on Lyme disease considered the abundance of ticks in their environment and the prevalence of *Borrelia burgdorferi* infection in ticks through comparison of kriging, allowing predictions of Lyme disease risk in humans (Nicholson & Mather, 1996). GIS and geospatial analysis may be extended to speciation events, and can be very useful in predicting movements of populations and species. Recent studies conducted on a hybrid zone, demonstrated the correlation between Mitochondrial DNA variation and geographical variations in bushcrickets (Ritchie et al, 2001) and the patterns and causes of geographic variation among their populations according to their environment (Kidd & Ricthie, 2000). In our study area, most of the species of warningly coloured butterflies and poisonous frogs are known to meet and hybridise with sibling species or with other morphological phenotype of the same species (Mallet, 1989, 1993; Shulte 1999; Joron 2000, 2001), and it could be interesting to examine structure of relationships between mimicry rings, and environmental variations by a geostatistical approach.

Fig 1a : Study area location (Tarapoto, Peru)

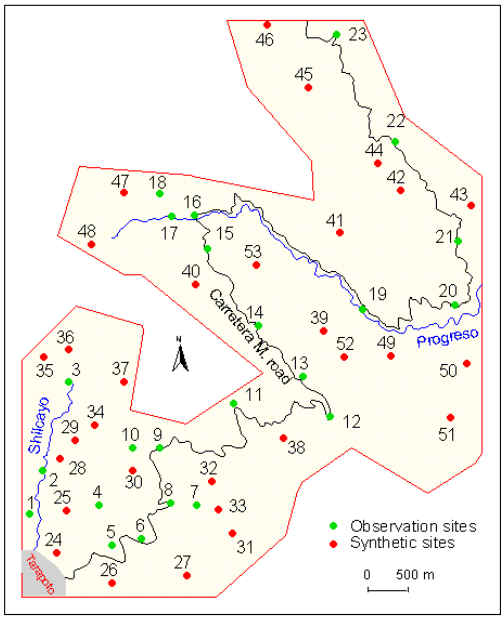

Fig. 1b : Tarapoto study area location and sites repartition

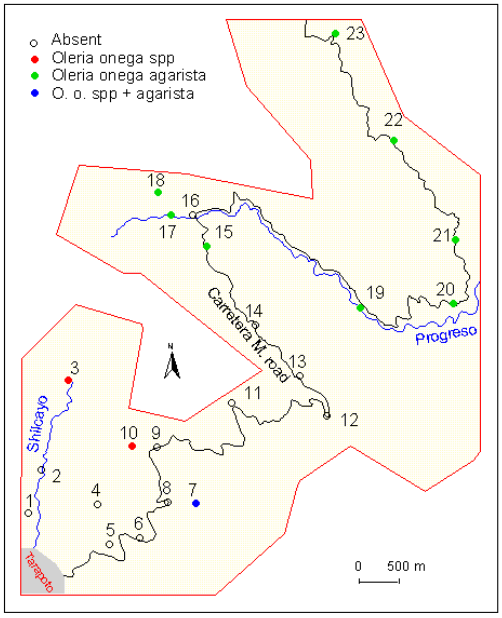

Fig 2a - Repartition of Oleria onega subspecies in sites

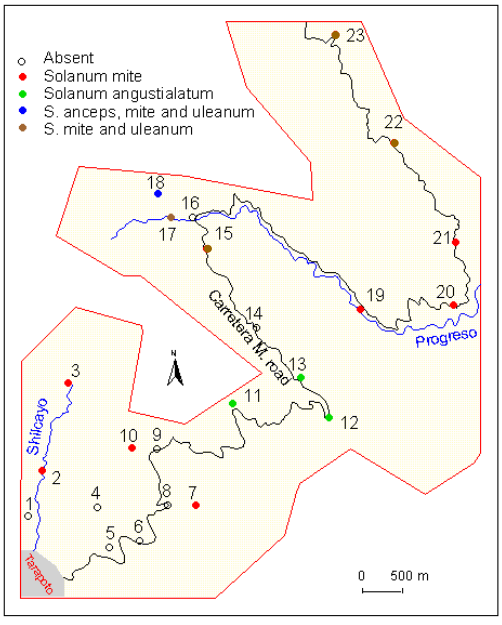

Fig 2b - Repartition of Solanum species in sites

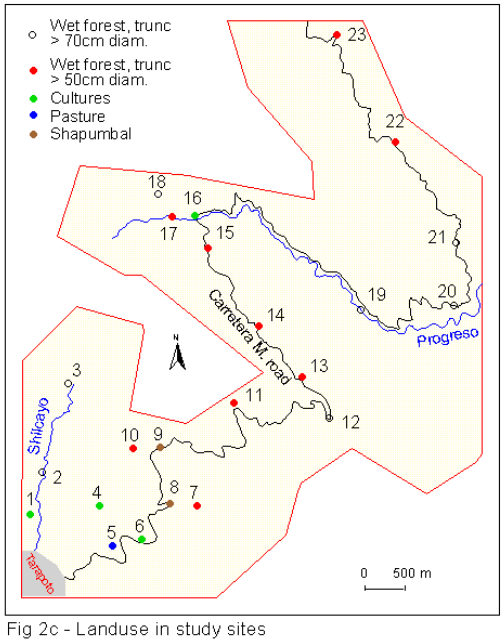

Fig 2c - Landuse in study sites

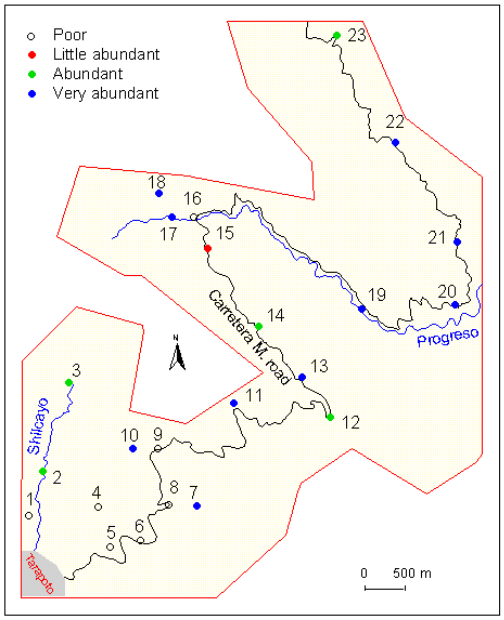

Fig 2d - Understorey abundance in sites

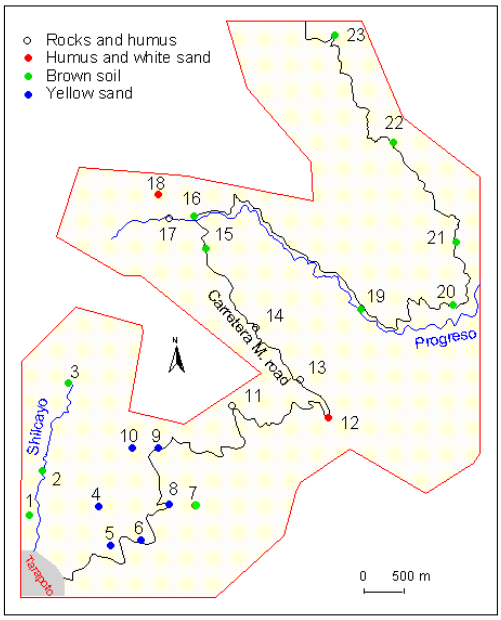

Fig 2e - Soil type in sites

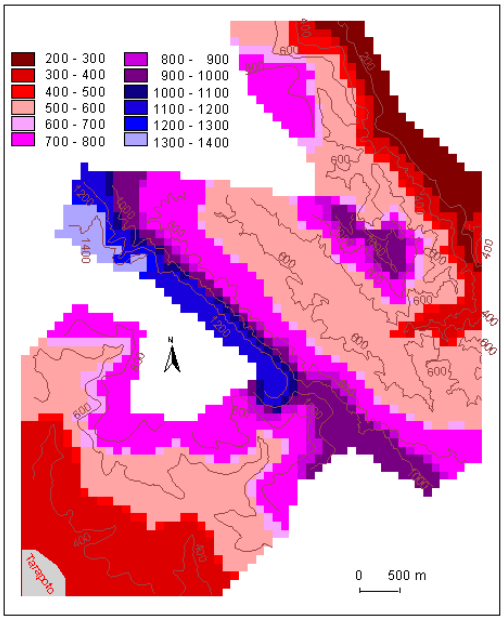

Fig 3 - Topography [m]

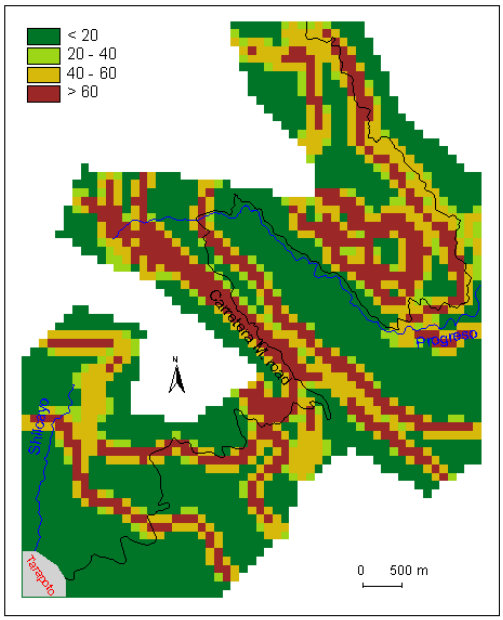

Fig 4 - Slopes in degrees

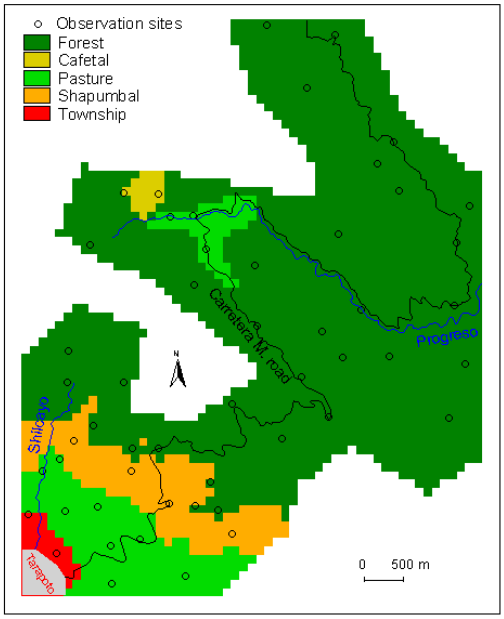

Fig 5 - Landuse

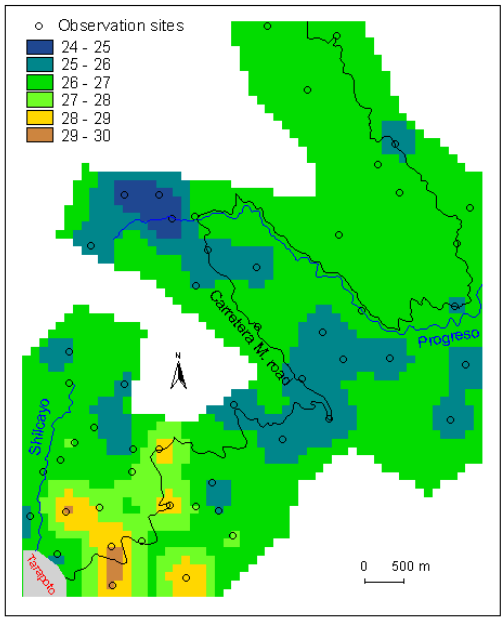

Fig 6a - Kriging of temperature [°C]

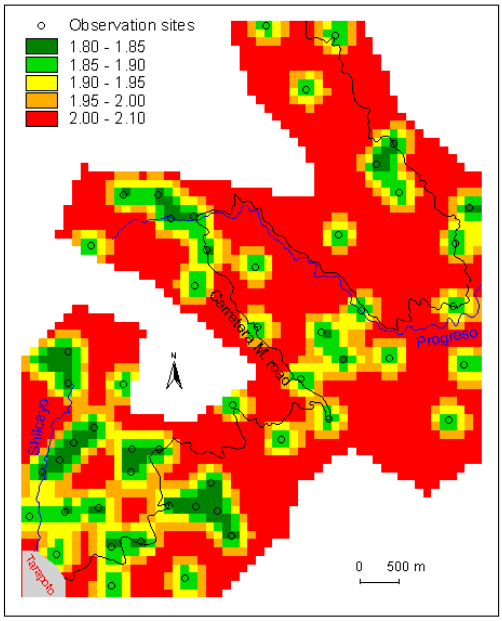

Fig 6b - Stand dev of temperature kriging

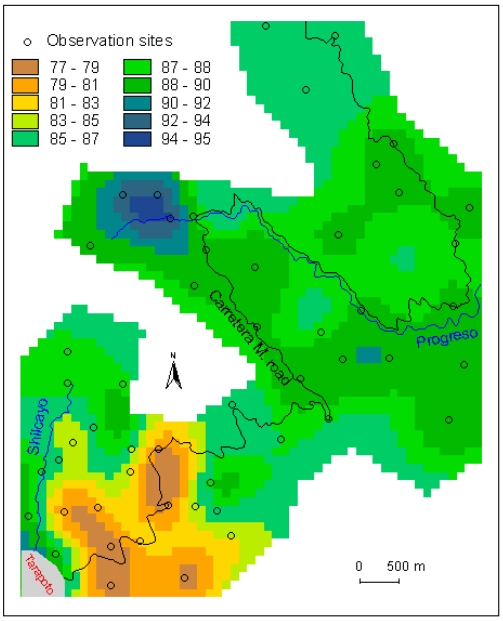

Fig 7a - Kriging of humidity [%]

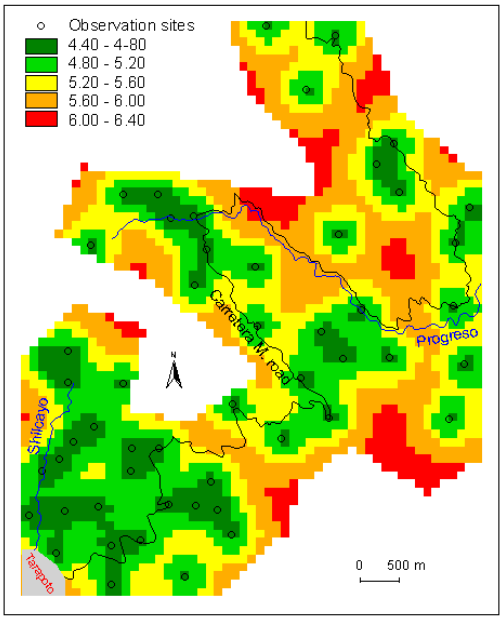

Fig 7b - Stand dev of humidity kriging

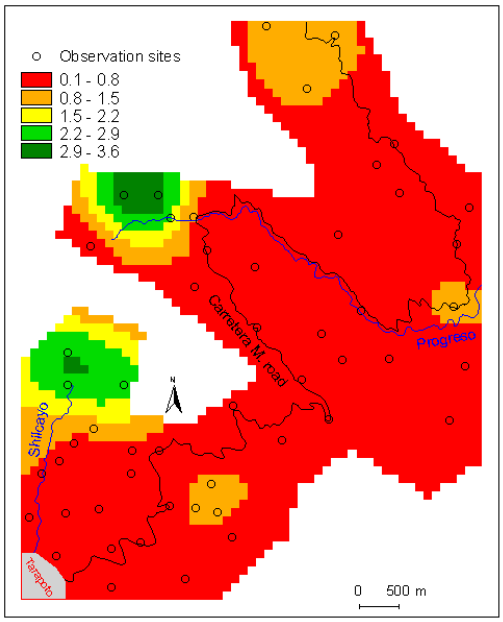

Fig 8a - Kriging of females number (mean value)

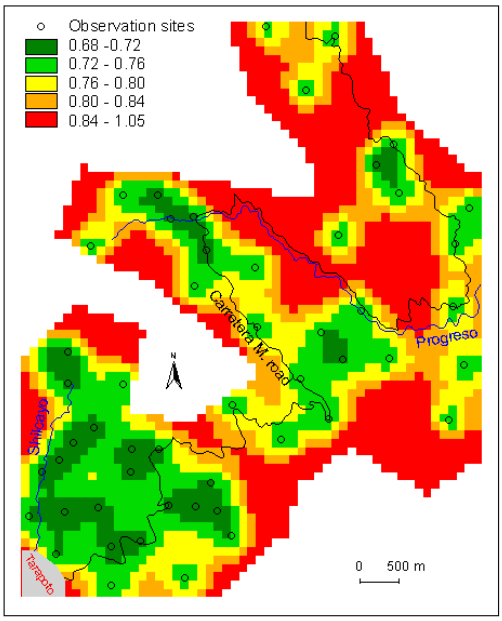

Fig 8b - Stand dev of females number kriging

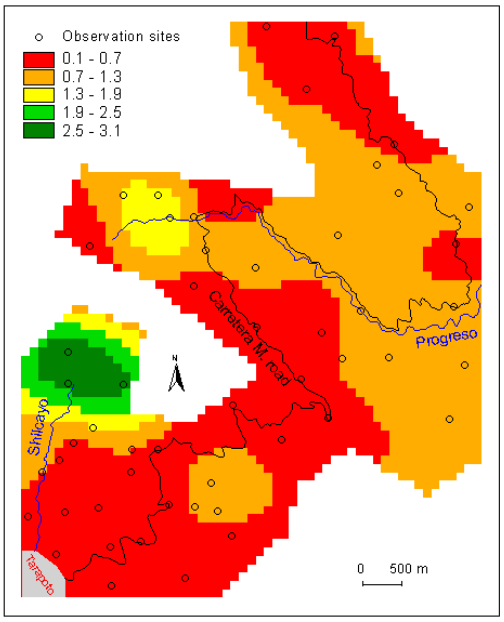

Fig 9a - Kriging of males number (mean value)

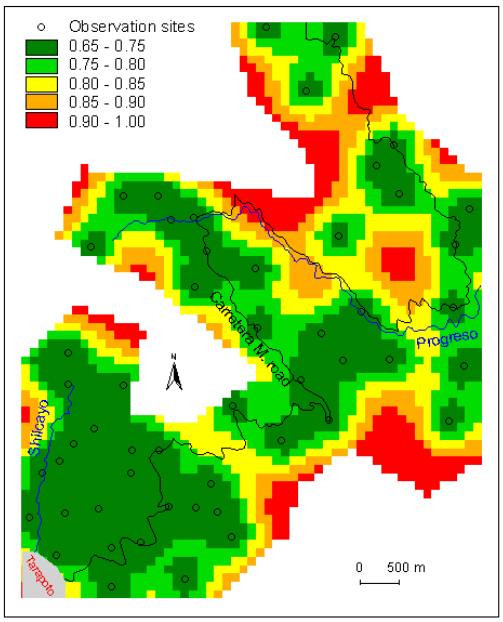

Fig 9b - Stand dev of males number kriging

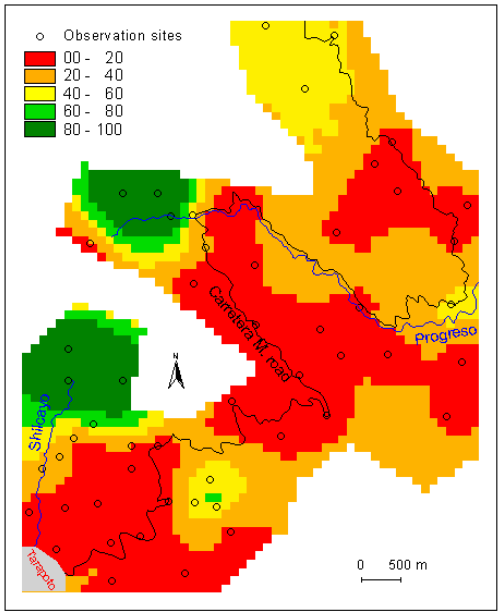

Fig 10 - probability to find 2 females (%)

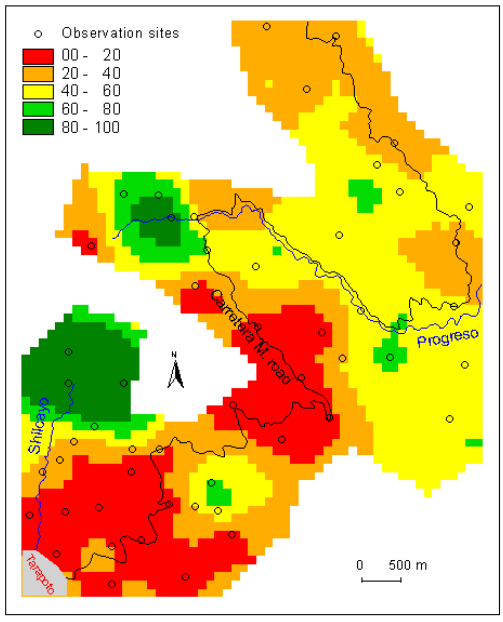

Fig 11 - probability to find 2 males (%)

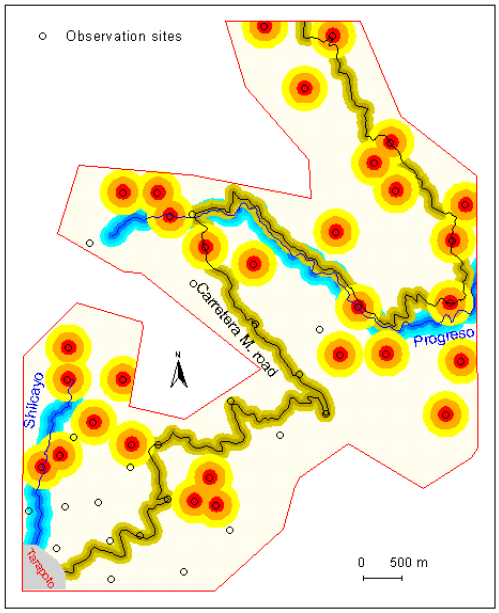

Fig 12 - Buffers of sites (200, 400, 600 m), roads (100, 200 m) and rivers (100, 200, 300 m)

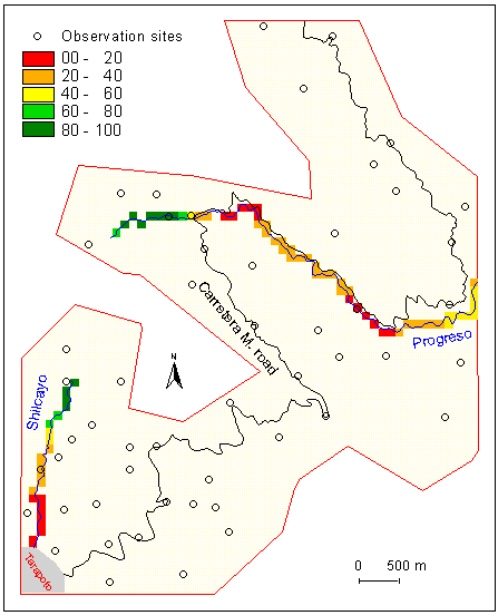

Fig 13 - Probability to find 2 females [%] within a zone of 100 m around rivers

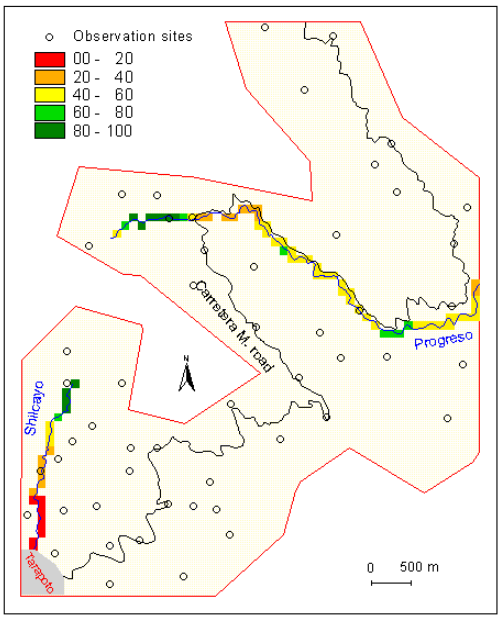

Fig 14 - Probability to find 2 males [%] within a zone of 100 m around rivers

CONCLUSION AND REMAINING QUESTIONS

The injection of synthetic data allowed us to obtain good interpolations of the different variables. The probabilities of the presence of butterflies, as well as a kriging of temperature and humidity were possible to estimate in inaccessible regions. However, we do not know how well these results fit. The results showed that the butterflies inhabit an altitude range between 300 and 800m. Their presence is correlated with temperature and humidity, regulated by the forest, but depends most of all on the presence of host plants. The Cerro Escalera constitutes a barrier not only for the butterflies but also for the host plants. The presence of the host plant species is correlated with soil quality and altitude. Through the kriging of temperature and humidity, we can observe that interactions between the butterfly populations on opposite slopes of the mountain may be possible. However the absence of the host plant on the upper part of the mountain may make such dispersal imposible.

Further studies should be done with more field observations and more data on the different sites, allowing a better establishment of the means of the different variables. Geographical distance should be correlated with genetical distances and estimation of the gene flow between populations, allowing a better understanding of dispersal pathways over or around the mountain.

Even so, this study remains a demonstration of the great possibilities to study the interactions between the environmental variables and organisms through a set of GIS and geospatial analysis techniques.

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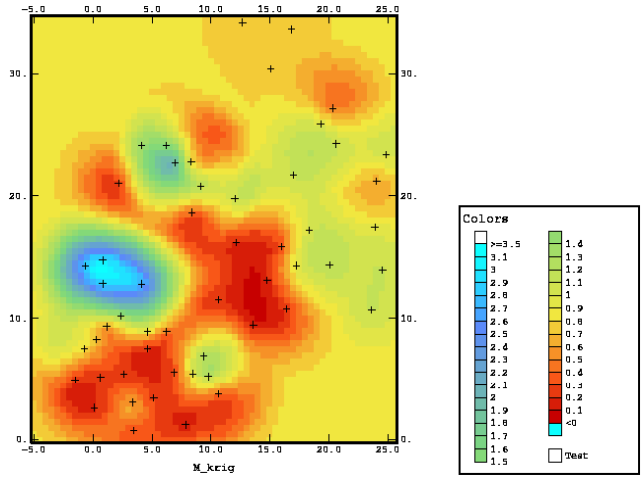
ANNEXES

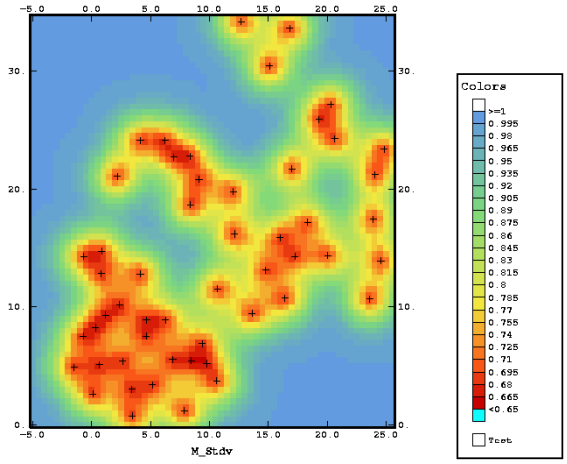
Annex 1 : Datas for interpolations

ID	X	Y	Mean T	Mean H	Mean F	Mean M
001	-01.510	04.894	026.00	091.30	000.00	000.00
002	-00.723	07.513	027.40	087.40	001.00	001.00
003	00.824	12.809	026.80	089.00	003.30	003.40
004	02.637	05.399	029.50	079.30	000.00	000.00
005	03.397	03.041	032.80	073.60	001.00	001.00
006	05.163	03.400	028.50	081.00	000.00	000.00
007	08.442	05.444	027.50	084.50	001.88	001.24
008	06.873	05.547	031.30	074.00	000.00	000.00
009	06.245	08.884	031.30	074.00	000.00	000.00
010	04.653	08.874	026.00	088.30	001.10	001.40
011	10.666	11.536	025.00	085.50	000.00	000.00
012	16.412	10.730	025.00	087.70	000.00	000.00
013	14.772	13.111	025.10	091.60	000.00	000.00
014	12.139	16.180	026.10	089.70	000.00	000.00
015	09.104	20.799	025.50	090.90	000.90	001.60
016	08.333	22.778	027.30	087.50	000.00	000.00
017	06.970	22.701	021.50	099.00	002.50	003.00
018	06.233	24.091	023.00	096.50	004.30	001.50
019	18.341	17.171	027.00	090.00	000.20	001.20
020	23.881	17.423	026.00	090.00	001.20	001.00
021	24.035	21.195	026.80	088.80	000.20	000.50
022	20.307	27.152	025.50	090.00	000.40	000.20
023	16.807	33.601	026.70	086.30	001.00	000.70
024	00.120	02.610	026.00	091.33	000.00	000.00
025	00.650	05.120	032.75	073.60	000.00	000.00
026	03.430	00.750	032.75	073.60	000.00	000.00
027	07.850	01.200	032.75	073.60	000.00	000.00
028	00.320	08.240	027.42	087.44	001.00	001.00
029	01.170	09.300	028.50	080.96	000.00	000.00
030	04.610	07.500	028.50	080.96	000.00	000.00
031	10.610	03.720	028.50	080.96	000.00	000.00
032	09.370	06.870	026.00	088.29	001.10	001.40
033	09.770	05.200	026.00	088.29	001.10	001.40
034	02.360	10.200	026.00	088.29	001.10	001.40
035	-00.660	14.270	026.00	088.00	003.30	003.40
036	00.830	14.720	026.00	088.00	003.30	003.40
037	04.130	12.790	026.00	088.00	003.30	003.40
038	13.600	09.460	025.00	087.70	000.00	000.00
039	15.990	15.860	025.00	087.70	000.00	000.00
040	08.390	18.640	026.07	089.70	000.00	000.00
041	16.990	21.710	027.00	090.00	000.20	001.20
042	20.620	24.250	027.00	090.00	000.20	001.20
043	24.820	23.350	027.00	090.00	000.20	001.20
044	19.290	25.890	027.00	090.00	000.20	001.20
045	15.090	30.410	026.67	086.33	001.00	000.70
046	12.680	34.170	026.67	086.33	001.00	000.70
047	04.130	24.120	023.00	096.50	004.30	001.50
048	02.170	21.050	025.08	091.60	000.00	000.00
049	20.040	14.350	025.50	092.00	000.20	001.20
050	24.540	13.910	025.50	092.00	000.20	001.20
051	23.610	10.670	025.50	092.00	000.20	001.20
052	17.240	14.290	025.50	092.00	000.20	001.20
053	12.020	19.780	025.50	092.00	000.20	001.20

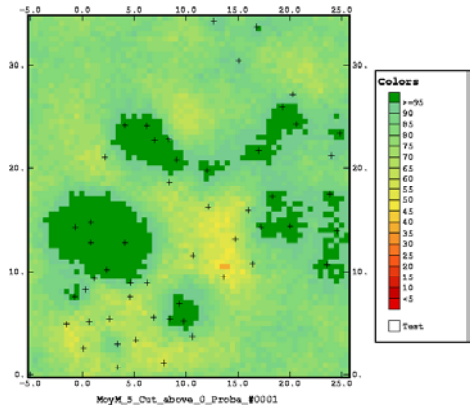
study sites Id 1 to Id 23 and synthetic sites id 24 to 53

ANNEX 2a : Kriging and kriging standard deviation of the mean number of males. (cell size 0.5x0.5 cm)

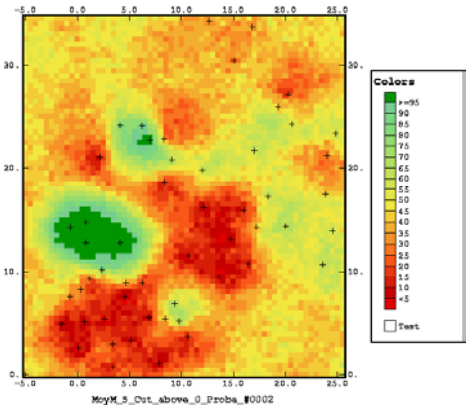




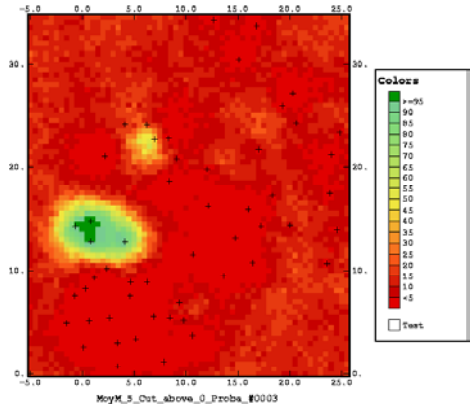
ANNEX 2b : Probabilities of males present per day from September to February (cell size 0.5x0.5 cm)

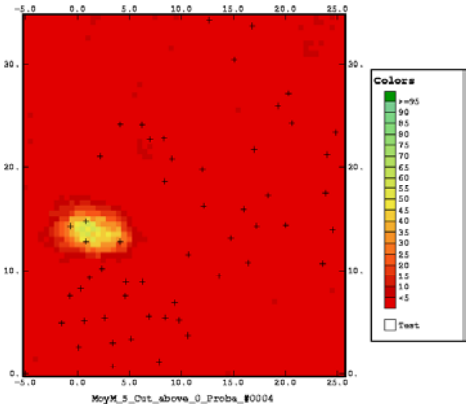


Probability of 1 male per day



Probability of 2 male per day





Probability of 3 male per day Probability of 4 male per day

Annex 3a : Quick statistics on data : Correlation Matrix (total number of samples = 53)

Variable	Temp_Moy (°)	Hum_percent	MoyF	MoyM
Temp_Moy (°)	1.00	-0.94	-0.38	-0.37
Hum_percent	-0.94	1.00	0.35	0.41
MoyF	-0.38	0.35	1.00	0.79
MoyM	-0.37	0.41	0.79	1.00

Moy = Mean, F = Female, M = Male, Temp = Temperature (°)
Hum_ percent = Humidity (%)

Annex 3b : Quick statistics on Kriging

Variable	Nb samples	Minimum	Maximum	Mean	Std. Dev.	Variance	Skewness	Kurtosis
T_krig	4340	24.00	30.02	26.84	0.50	0.25	1.89	15.32
T_stdv	4340	1.79	2.10	2.05	0.08	0.01	-1.65	4.61
H_krig	4340	77.14	95.69	87.35	2.22	4.91	-1.63	8.81
H_stdv	4340	4.44	6.09	5.66	0.50	0.25	-0.83	2.31
F_krig	4340	-0.00	3.69	0.72	0.56	0.31	2.38	9.64
F_stdv	4340	0.68	1.06	0.91	0.12	0.01	-0.17	1.56
M_krig	4340	0.08	3.12	0.82	0.43	0.19	1.97	9.82
M_stdv	4340	0.66	1.00	0.87	0.11	0.01	-0.26	1.60

krig = kriging, stdv = standard deviation, T = Temperature, H = Humidity, F = Female, M = Male,

Annex 3c : Quick statistics on Kriging

Variable	Nb samples	Minimum	Maximum	Mean	Std. Dev.	Variance	Skewness	Kurtosis
Simu_MoyF_Me	4340	0.12	3.31	0.86	0.33	0.11	2.41	12.49
Simu_MoyF_D	4340	0.96	2.81	1.81	0.28	0.08	0.21	2.98
Simu_MoyF_L	4340	2.55	8.30	4.26	0.68	0.46	0.73	4.11
Simu_MoyF_S	4340	-5.38	0.80	-2.50	0.66	0.43	0.08	4.33
Simu_MoyM_Me	4340	-0.04	3.74	0.70	0.57	0.32	2.31	9.33
Simu_MoyM_D	4340	0.18	1.76	0.78	0.31	0.09	0.09	1.92
Simu_MoyM_L	4340	0.95	5.87	2.90	0.82	0.67	0.35	2.78
Simu_MoyM_S	4340	-3.84	2.46	-1.49	0.79	0.62	1.14	6.83

Simu = simulation, Moy = mean, F = Female, M = Male, Me = mean, D = display, S = smallest, L = largest.

CONCLUSION and OUTLOOK

CONCLUSION AND OUTLOOK

1. *Are the four Solanum species host plants of Oleria onega agarista and O. o.* ssp.*? How does oviposition behaviour of the two Oleria subspecies differ?* The most important result of the experiments conducted in the cages is that *Solanum mite* is the most preferred host plant of both subspecies. Switches to other *Solanum* species occur when *S. mite* is rare or absent. *O. o.* ssp. which lives on the SW slope of the mountain, lays most of its eggs on objects close to the plant, whereas *O. o. agarista,* which lives on the NE slope, laid them on the plant itself. A possible explanation may be that the risk of predation on eggs when laid on the host plant is higher on the SW slope and that this selected for a new oviposition strategy. The cost of oviposition away from the plant may be compensated by a higher survival rate of eggs and by the higher host plant density found on the SW side of the mountain

2. *Is oviposition preference correlated with preference and performance of the larvae? Is there a difference in potential predation pressure on larvae of both subspecies, that may affect their behaviour? Is there differences in potential predators species for different oviposition environments?* Among the four host plants tested, *Solanum mite* was the most preferred host by the larvae and preference among species was highly correlated with larval performance. Larvae reared on *S. mite* were bigger and survival was greater on this plant than on *S. anceps.* Therefore female and larval choices were highly correlated with each other and with larval performance.

   Comparing the larval behaviour of both *Oleria* subspecies, we found that *O. o. agarista* larvae move more than the these of *O. o.* ssp. larvae. This difference may be related to the fact that this subspecies suffers less predation than the other, but also that *S. mite* is less abundant on the NE slope and that larvae would have to wander more to find the host plants if laid on other substrates. When eggs were artificially deposited in the field, the eggs placed on the SW slope showed a higher survivorship when laid on other objects than on the host plant. When host plant stems, were protected by sticky glue in the SW slope, egg disappearance was significantly higher on non- protected plants. Taken together, these observations led to the hypotheses that *O. o.* ssp. might suffer more predation in its narural environment than does *O. o. agarista*, and

therefore that predation was higher on the SW slope. In the field, 70-80% of the potential predators collected on both slopes of the mountain were ants. Among them, the only abundant genus of predators that was found only on the SW slope and not on the NE side was the Ponerinea genus *Ectatomma*. We cannot assert that *Ectatomma* are the ultimate cause of oviposition behaviour of *O. o.*ssp.*,* as no acts of predation were observed when eggs were offered to the ants. However, we observed that *Ectatomma* individuals spend most of their time on plants (*Solanum spp.* and others), that not only are they present on the SW slope only, but also abundant there, and that they are considered as potential predators in the literature. Therefore we suggest that they may constitute a selective pressure, either because of predation on eggs, or because they might repel ovipositing females, leading to the behaviour of laying eggs next to the host plant.

3. *Are the two Oleria subspecies genetically distinct ?* The results showed that the two subspecies are distinct and that together they are well separated from the outgroup. Hybrids are intermediate between subspecies, but more closely related to some populations near the top of the mountain. These populations still show traits of probable hybridisation events in the past. Genetic variation was higher among populations than between them. Nevertheless, we believe that gene flow had occurred between them in the recent past (max. ten years ago). On both slopes, selective pressures had led the rare forms to evolve to the more common pattern, and the only population where hybrids seem to suffer less constraint is Estero, near Shapaja.

4. *Is there a relationships between distribution of Oleria and environmental factors such as altitude, temperature, humidity and other factors?* The results have shown that the butterflies inhabit an altitude range between 300 and 800m. Their presence is correlated with temperature and humidity, regulated by the forest cover, but depends most of all on the presence of host plants. The abiotic conditions in which the butterflies were the most abundant were at temperatures between 26-27°C and relative humidity between 85-90%. The Cerro Escalera constitutes a barrier not only for the butterflies but also for the host plants. The presence of the different host plant species is correlated with soil quality (sand, clay, rocks) and altitude. Through the kriging of temperature and humidity, we can observe that interactions between butterfly populations on the

two slopes the mountain may be possible. However, the absence of host plants on the upper part of the mountain make this unlikely. The use of synthetic data allowed us to conduct interpolations of the different variables. The probabilities of butterfly presence, as well as kriging of temperature and humidity then permitted estimation in inaccessible region. However we do not know how well these results fit with reality, and they remain a hypothesis to be confirmed in the filed. Even so, this study serves as a demonstration of the great opportunities to study the interactions between the environmental variables and studied organisms through a set of GIS and geospatial analysis techniques.

Based on the results presented, a set of new questions arose :

1. To define better the suitability of the four host plant for the larvae, it would be interesting to conduct an analysis of their chemical compounds to determine whether they emit different volatiles that may affect the female choice.
2. The relationships between ants and butterflies need further observations. Predation events on eggs and larvae should be observed in the field as well as the possible influence of ants on ovipositing females. The extrafloral nectaries appear not to occur in the studied *Solanum* species and the relation *Ithomiinae-Solanum-Ecatomma* need further study. One case has already been cited in the literature of possible mutualistic interactions between *Ectatomma* ants and *Mechanitis isthmia* (Ithomiinae) (Young, 1978) in which the larvae provide exsudates to the ants when feeding on *Solanum* sp.
3. Results obtained with molecular markers revealed that further studies with other markers are required to assert the real taxonomical statute of the two subspecies, and to measure traits of hybridisation degree between them. Further studies should include other *Oleria* species present in the area (*O. lerida lerida* and *O. gunilla serdolis*), focusing also on the possible hybridization with the two forms of *O. lerida lerida*.
4. Another interesting point with genetic studies is a better understanding of the loci coding for the colour-pattern differences, for oviposition behaviour and differences between behavioural ability and define if one of the pattern is dominant over the others.

5. The relations between butterflies and their environmental parameters requires further study with more field observation and more data on the different sites, allowing a better establishment of the means of the different variables. Correlation between geographical distance and genetical distances should be examined, as well as gene flow between populations, allowing a better understanding of the dispersal pathways that exist between populations on the two slopes of mountain. Studies of Orthoptera have indicated that GIS and molecular results taken together might help us to understand speciation processes (Kidd & Ritchie, 2000; Ritchie et al. 2001).

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EDUCATION :

1979-1988	Primary and secondary school at the "Ecole Rudolf Steiner", of Lausanne.
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1991	" Baccalauréat scientifique " (exams in Mathematics, Physics, Biology, Chemistry, Geography, History, German, English, French).
1992-1996	Studies in Biology at the University of Neuchâtel, Switzerland.
oct. 1996	"Diplôme" in biology with specialisation in Entomology. Title of Diploma thesis : " Etude des différences morphométriques entre populations d'*Oreina cacaliae*, *speciosissima* et *elongata* (Chrysomelidae)" (Spatial morphological differentiation of the three species) with Prof. Martine Rahier.
1997-2001	PhD thesis at the University of Neuchâtel.(Prof. Martine Rahier): Biology, Behaviour and Taxonomy of two Ithomiinae butterflies: *Oleria onega agarista and Oleria onega ssp.* " in collaboration with Dr. James Mallet, University College, London, and Dr. Gerardo Lamas, Universidad Nacional Mayor de San Marcos, Lima, Perú.

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1991-1992	Travel in South America : Argentina, Paraguay, Peru, Bolivia and Colombia.
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	-Conections and visit at the entomological collections of the Universidad del Valle, Cali, Colombia.
	Field work :
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apr. 1997	-with students in Iquitos (with the Universidad Nacional de la Amazonia peruana),
	Teaching:

1997-2001	Participation in and organisation of practical courses in Entomology at the University of Neuchâtel, with second year students.
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PUBLICATIONS:

- Oviposition behaviour and host-plant preferences of *Oleria onega agarista* and *Oleria onega ssp.* in north-eastern Peru (Ithomiinae, Nymphalidae), In prep.
- Genetic (RAPD) diversity between *Oleria onega agarista* and *Oleria onega ssp*. (Ithomiinae, Nymphalidae) in north-eastern Peru. In prep.
- Larval performance, and predation effect of ants on two Ithomiinae (Nymphalidae) butterfly subspecies. In prep.