

SELECCIÓN NATURAL SOBRE
A COR DAS FLORES DE
Gentiana lutea L.



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SANTIAGO DE COMPOSTELA

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TESE DE DOUTORAMENTO

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Asdo.:

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CERTIFICAN

Que son directores da Tese de Doutoramento titulada «Selección natural sobre a cor das flores de *Gentiana lutea* L.» presentada por Tania Veiga Blanco, aluna do Programa de Doutoramento 2116-08-1V01 «Biodiversidade de Conservación de Medio Natural»; e autorizan a presentación da tese indicada, considerando que reúne os requisitos esixidos no artigo 34 do regulamento de Estudos de Doutoramento, e que como Directores da mesma non incorren nas causas de abstención establecidas na lei 30/1992.



Santiago de Compostela a 25 de outubro de 2014

D. Javier Guitián Rivera

Dña. Mar Sobral Bernal



Resúltame difícil seleccionar ás persoas que considero que colaboraron na realización deste traballo. Si o bater das ás dunha bolboreta na Galiza, podería provocar unha tormenta en Pernambuco; penso que tódalas persoas que se cruzaron no meu camiño, influenciaron este traballo doutoral. Mais por non utilizar nin un tempo que podería ser infinito, nin eucaliptos de máis, espero que con estas liñas o meu agradecemento se faga todo o universal que deba ser.

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ABSTRACT

Floral variation within species may result from the spatial variation of selective agents. Flower color variation might be shaped by the action of animals feeding on the plant species. Pollinators might exert natural selection on color if flower color is related to their foraging efficiency. In addition, non-pollinator agents such as predispersal seed predators may select on flower color, if color indicates food resources (seeds) or if color is related to deterrent compounds. Thus, flower color variation among plant populations might reflect adaptation to local conditions such as the interacting animal community. First, we investigated the spatial variation in floral and vegetative traits among 12 populations of *Gentiana lutea* located across the western end of its distribution. Populations differed in floral and vegetative traits and this variation was geographically structured. Floral variation –in particular flower color– drags phenotypic variation in *G. lutea*. Second, we addressed selection on flower color within a *Gentiana lutea* population; we found that selection acts on flower color, mediated by both pollinators and seed predators. Both agents favored yellow-flowered individuals, thus opposed selection by pollinators and seed predators does not explain flower color variation within this population. Third, we inspected the variation in selective pressures on flower color among 12 *G. lutea* populations. We found that selective pressures explain part of the flower color differences among populations in *G. lutea* and pollinators play a role on flower color local adaptation.

KEYWORDS

Gentiana lutea; geographic color variation; natural selection; pollinators; seed predators.

RESUMO

A variación floral dentro dunha de especie pode resultar da variación espacial nos axentes de selección. A cor floral podería variar pola selección dos animais que se alimentan das plantas. Os polinizadores poderían exercer selección natural sobre a cor, si a cor inflúe na eficacia do consumo. Outros axentes de selección, como os predadores de sementes, poderían seleccionar sobre a cor, si a cor indica recursos alimentarios (sementes) ou si a cor está relacionada con defensas químicas. Así, a variación na cor das flores entre poboacións podería reflectir adaptación a condicións locais como a comunidade de interactuantes. En primeiro lugar, investigamos a variación espacial en atributos florais e vexetativos en 12 poboacións de *Gentiana lutea* localizadas no extremo occidental da súa distribución. Os nosos resultados indicaron que, maila que as poboacións difiren en atributos vexetativos, son os caracteres florais –particularmente a cor das flores– os que arrastran a variación fenotípica en *G. lutea*. En segundo lugar, estudamos a selección sobre a cor nunha poboación de *G. lutea*; atopamos selección sobre a cor, exercida por polinizadores e predadores de sementes. Ambos axentes favoreceron individuos con flores amarelas, polo que nesta poboación, a variación na cor non se pode explicar como resultado dunha selección oposta exercida por polinizadores e predadores. En terceiro lugar, inspeccionamos a variación nas presións de selección sobre a cor en 12 poboacións de *G. lutea*. Atopamos que as presións selectivas explican parte das diferenzas na cor das flores entre poboacións de *G. lutea* e que os polinizadores inflúen na adaptación local da cor.

PALABRAS CHAVE: *Gentiana lutea*; variación xeográfica na cor; selection natural; polinizadores; predadores de sementes.

RESUMEN

La variación floral dentro de una especie puede provenir de la variación espacial en los agentes de selección. El color floral podría variar por la selección de los animales que se alimentan de las plantas. Los polinizadores podrían ejercer selección natural sobre el color, si el color se relaciona con la eficacia en el forrajeo. Otros agentes de selección, como los predadores de semillas, pueden seleccionar sobre el color, si el color indica recursos alimentarios (semillas) o si está relacionado con defensas químicas. Así, la variación en el color entre poblaciones podría reflejar adaptación a condiciones locales como la comunidad de interactuantes. En primer lugar, investigamos la variación espacial en rasgos florales y vegetativos en 12 poblaciones de *Gentiana lutea* localizadas en el extremo occidental de su distribución. Nuestros resultados indican que, aunque las poblaciones difieren en rasgos vegetativos, son los florales –particularmente el color– los que arrastran la variación fenotípica en *G. lutea*. En segundo lugar, estudiamos la selección sobre el color en una población de *G. lutea*; encontramos selección sobre el color, ejercida por polinizadores y predadores de semillas. Ambos agentes favorecieron individuos con flores amarillas; en esta población, la variación en el color no proviene de una selección opuesta ejercida por polinizadores y predadores. En tercer lugar, inspeccionamos la variación en las presiones de selección sobre el color en 12 poblaciones de *G. lutea*. Encontramos que las presiones selectivas explican parte de las diferencias de color entre poblaciones de *G. lutea* y que los polinizadores influyen en la adaptación local del color.

PALABRAS CLAVE: *Gentiana lutea*; variación geográfica en el color; selección natural; polinizadores; predadores de semillas.



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1. INTRODUCCIÓN XERAL



As flores son os órganos reprodutivos das anxiospermas que constitúen os reclamos para os polinizadores e suministran recompensas (Fenster et al. 2004). O papel selectivo que os polinizadores xogan sobre as características das flores e a súa importancia como axentes na diversificación, constitúe un tema recorrente na bioloxía evolutiva dende Darwin (1862) e numerosos estudos analizaron a selección mediada polos polinizadores sobre os atributos florais, tanto en condicións naturais (Campbell et al. 1991, Herrera 1993, Maad 2000) como artificiais (Herrera 2001, Aigner 2004, Castellanos et al. 2004, Campbell 2009). Ademais dos polinizadores, as plantas interaccionan con animais antagonistas; é dicir, animais que extraen recursos pero non polinizan e ademais causan danos (revisado en Strauss e Whittall 2006).

Dende unha perspectiva máis ampla, realizouse un importante esforzo para analizar o balance entre mutualistas (polinizadores e dispersantes) e antagonistas (herbívoros e predadores de sementes) sobre a ecoloxía reprodutiva das plantas (Strauss 1997). Experimentos de campo, manipulando a presenza de polinizadores e herbívoros, mostraron que o impacto negativo dos herbívoros varía en función da presenza ou ausencia dos polinizadores (Herrera 2000, Herrera et al. 2002, Sánchez-Lafuente 2002) e viceversa (Gómez 2003). Estes e outros resultados mostraron, unha vez máis, que as flores son estruturas de «compromiso», suxeitas non só ás presións selectivas dos polinizadores, senón tamén ás de organismos antagonistas e de factores abióticos, de xeito que estas presións poden ser tan ou máis intensas que as exercidas polos polinizadores e, ademais, de signo contrario (Brody 1997, Galen 2000, Herrera 2000, Adler et al. 2001, Ashman 2002, Strauss et al. 2004, Strauss e Whittall 2006 e referencias alí citadas).

Neste contexto, a cor das flores resulta interesante para estudar a evolución das especies porque alén de variar e herdarse (Bradshaw e Schemske 2003, Fenster et al. 2004, Lee 2007, Rausher 2008), diversos axentes poden afectar a fitness a través da cor e así exercer presións selectivas sobre a cor (Clegg e Durbin 2000, Strauss e Whittall 2006, Rausher 2008). A cor das flores se ten considerado tradicionalmente como un atributo clave á hora de atraer certos tipos de polinizadores (Grant 1950, Rausher 2008). Varios estudos teñen demostrado que os polinizadores prefiren morfos de determinadas cores (Bradshaw e Schemske 2003, Irwin e Strauss 2005) debido a que difiren nos seus sistemas visuais, o cal afecta á detectabilidade (Chittka e Waser 1997, Rodríguez-Gironés e Santamaría 2004). Por exemplo, as abellas detectan a luz UV pero teñen dificultades para percibir cores vermellas, e as aves teñen receptores para a luz vermella. Un cambio na cor das flores pode beneficiar a unha planta se aumenta a detección polos polinizadores máis eficientes e limita a entrada dos visitantes indesexados (Rodríguez-Gironés e Santamaría 2004).

Sen embargo, os resultados acerca da selección da cor polos polinizadores foron recentemente cuestionados, entre outros motivos, debido á imposibilidade de separar os efectos da selección sobre a cor, dos efectos sobre outros caracteres xenéticamente ligados (Rausher 2008). Por exemplo, as encimas necesarias para a síntese de antocianinas –un subgrupo de flavonoides que interveñen na cor das flores– tamén participan na síntese doutros compostos implicados na protección fronte a toxicidade do solo (Ma et al. 2001, Yoshida et al. 2003, Yoshida et al. 2006), fronte o estrés térmico (Coberly e Rausher 2003) e na defensa química fronte a herbívoros (Armbruster 2002, Irwin et al. 2003, Strauss et al. 2004, Strauss e Whittall 2006, Lev-Yadun e Gould 2009). Así, tanto factores abióticos coma animais antagonistas poden ser axentes de selección natural sobre a cor das flores.

Especificamente, os predadores de sementes poden destacar como axentes selectivos sobre a cor das flores porque a cor podería indicar por un lado a cantidade de recursos alimentarios para as larvas (se, por exemplo, a cor inflúe no éxito de polinización), e por outro lado a presenza de compostos secundarios tóxicos (se existe pleiotropía; Irwin et al. 2003). Ademais, o impacto sobre a produción de sementes é directa (Kolb et al. 2007). Deste modo, os predadores de sementes poderían seleccionar as plantas segundo os atributos florais (Frey 2004, Kolb et al. 2007, Caruso et al. 2010), podendo anular o efecto dos polinizadores (se as flores máis polinizadas son as máis consumidas; ou sexa, se comparten preferencias) ou reforzalo (se as plantas máis polinizadas son as menos consumidas; ou sexa, se posúen preferencias opostas).

Na actualidade, a variación espacial das interaccións ecolóxicas entre plantas e animais constitúe un aspecto central no estudo das consecuencias evolutivas de tales procesos. Desde a formulación explícita da hipótese do mosaico xeográfico (Thompson 1994) numerosos estudos incorporaron a variación espazo-temporal á hora de analizar as consecuencias das interaccións, tanto mutualistas como antagonistas (Thompson 1997, Benkman 1999, Gómez e Zamora 2000; Benkman et al. 2001, Parchman e Benkman 2002, Stinchcombe e Rausher 2002, Zangerl e Berenbaum 2003, Rey et al. 2006). No caso concreto dos polinizadores e os herbívoros, pártese da premisa de que si a magnitude e dirección das respectivas presións de selección difiren entre poboacións, danse condicións para que existan diferentes traxectorias evolutivas en cada unha delas (Thompson 1994, Gómez e Zamora 2000). Comprender axeitadamente as consecuencias dos efectos de polinizadores e herbívoros implica, por tanto, incorporar o contexto xeográfico no estudo das interaccións (Gómez e Zamora 2000, Rey et al. 2006 e referencias nestes traballos).

A variación xeográfica nos atributos florais (cor, tamaño, forma, etc.) dentro da mesma especie e entre especies próximas, foi documentada en numerosas ocasións (Mascó et al. 2004, Herrera et al. 2006, Medrano et al. 2006, Harder e Johnson 2009, Arista et al. 2013). Esta variación pode orixinarse por diferentes mecanismos tanto aleatorios, como mutacións e deriva xenética, coma non aleatorios, como a selección natural diverxente –a causada polos diferentes escenarios que xeran polinizadores, herbívoros e outros factores de selección bióticos e abióticos (ver Herrera et al. 2006)– ou a plasticidade fenotípica en resposta á heteroxeneidade ambiental (Price et al. 2003, Mitchell-Olds et al. 2007).

Diferentes estudos mostraron a relación entre a variación espacial das características das flores e a identidade ou morfoloxía dos polinizadores (Robertson e Wyatt 1990, Johnson e Steiner 1997, Gómez et al. 2008) e herbívoros (Gómez e Zamora 2000, ver Strauss e Whittall 2006). De xeito concreto, a variación espacial na cor floral pode reflexar adaptación local e simultaneamente, promover a especiación ó reducir o fluxo de xenes entre morfos, asociado a cambios nos polinizadores (Schemske e Bradshaw 1999; Hodges et al. 2002; Bradshaw e Schemske 2003).

A pesar de que son numerosos os estudos que abordan a variación nas características florais entre poboacións e a súa relación cos polinizadores, a maioría destes estudos non proban explícitamente a existencia de adaptación local. Recentemente, Herrera et al. (2006) deseñan un protocolo explícito de análise da variación espacial na selección para demostrar que polinizadores e/ou herbívoros son responsables da variación floral nunha especie. Con este obxectivo, propoñen unha aproximación estruturada en cinco pasos: 1) Caracterizar a variación xeográfica nos polinizadores e/ou herbívoros, 2) Demostrar que existe selección polos polinizadores e/ou herbívoros sobre os atributos florais, 3) Demostrar a diverxencia xeográfica na

selección, 4) Evaluar o axuste entre a selección e a diverxencia fenotípica e, 5) Mostrar que as diferenzas entre poboacións nos caracteres florais teñen base xenética.

O propósito deste traballo é investigar si as presións de selección locais, explican a variación xeográfica da cor en *Gentiana lutea*, e estudar o papel de polinizadores e predadores de sementes como axentes de selección natural sobre esta especie. En primeiro lugar, alén de describir a historia natural da especie, describimos con detalle o sistema de medida e cuantificación da cor e estudamos o sistema reprodutivo (capítulo 2). En segundo lugar, para coñecer que atributos poden estar baixo selección, describimos e comparamos a variabilidade entre poboacións de caracteres florais e vexetativos e a súa relación coa variación en factores abióticos (capítulo 3). En terceiro lugar documentamos a selección directa e indirecta dos polinizadores e predadores de sementes sobre a cor (capítulo 4). E xa por último, estudamos se existe variación espacial na selección e si a cor floral está adaptada localmente a estas presións de selección (capítulo 5).

Gentiana lutea adecúase ó estudo da selección natural sobre a cor das flores debido a que: (1) a cor floral en *G. lutea* posúe base xenética (Zhu et al. 2002, 2003); (2) posúe variabilidade tanto entre poboacións (indicativo de adaptación local) coma dentro das poboacións (necesaria para que actúe a selección natural); (3) depende de polinizadores para producir sementes (Rossi 2011) e (4) os froitos son atacados por predadores de sementes (Kóstic et al. 2006). Por outra banda, na área de estudio (Cordillera Cantábrica na península Ibérica) varía a comunidade de polinizadores (Obeso 1992, Ploquin et al. 2013) o que aumenta a potencialidade destes animais como axentes de selección sobre a cor das flores de *G. lutea*. Consideramos que a variación espacial na interacción con estes mutualistas e antagonistas pode provocar, mediante selección fenotípica, as diferenzas na cor da flores entre poboacións.

A pesar da importancia evolutiva da cor das flores (Rausher 2008) e do seu vínculo claro con diversos axentes de selección, non abundan estudos que combinen o rol de polinizadores e animais antagonistas (algúns exemplos son: Frey 2004, Streisfeld e Kohn 2007, Caruso et al. 2010, Carlson e Holsinger 2013). Tampouco abonda a investigación de hipóteses alternativas como a adaptación a factores abióticos, tanto edáficos como climáticos, ou a selección natural indirecta sobre a cor das flores (Frey 2007, Campbell 2009). Son raros os traballos que abarcan diferentes poboacións naturais e un rango xeográfico amplo (Whibbley et al. 2006, Streisfeld e Kohn 2007, Carlson e Holsinger 2013). Así, ata o momento, este é o único estudo sobre selección natural na cor das flores que contempla o rol simultáneo dos polinizadores e dos depredadores de sementes sobre a variación xeográfica da cor, tendo ademais en conta o posible efecto dos factores abióticos e o papel da selección indirecta que acontece a través dos atributos fenotípicos correlacionados.

1.1 REFERENCIAS

- Adler, L. S., R. Karban, e S. Y. Strauss. 2001. Direct and indirect effects of alkaloids on plant fitness via herbivory and pollination. *Ecology* 82:2032-2044.
- Aigner, P. A. 2004. Floral specialization without trade-offs: optimal corolla flare in contrasting pollination environments. *Ecology* 85:2560-2569.
- Arista, M., M. Talavera, R. Berjano, e P. L. Ortiz. 2013. Abiotic factors may explain the geographical distribution of flower colour morphs and the maintenance of colour polymorphism in the scarlet pimpernel. *Journal of Ecology* 101:1613-1622.
- Armbruster, W. S. 2002. Can indirect selection and genetic context contribute to trait diversification? A transition-probability study of blossom-colour evolution in two genera. *Journal of Evolutionary Biology* 15:468-486.

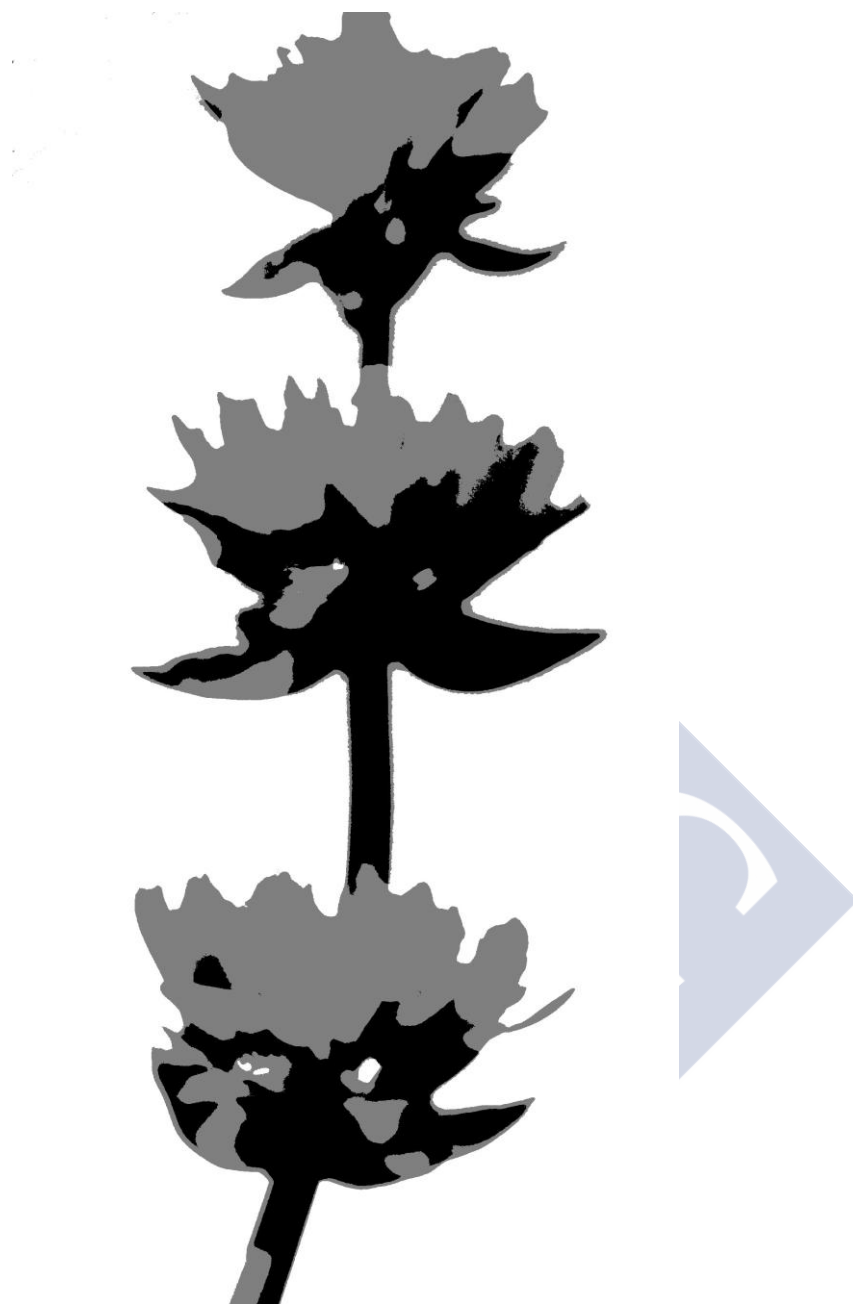
- Ashman, T. 2002. The role of herbivores in the evolution of separate sexes from hermaphroditism. *Ecology* 83:1175-1184.
- Benkman, C. W. 1999. The selection mosaic and diversifying coevolution between crossbills and lodgepole pine. *The American Naturalist* 153:S75-S91.
- Benkman, C. W., W. C. Holimon, e J. W. Smith. 2001. The influence of a competitor on the geographic mosaic of coevolution between crossbills and lodgepole pine. *Evolution* 55:282-294.
- Bradshaw, H., e D. W. Schemske. 2003. Allele substitution at a flower colour locus produces a pollinator shift in monkeyflowers. *Nature* 426:176-178.
- Brody, A. K. 1997. Effects of pollinators, herbivores, and seed predators on flowering phenology. *Ecology* 78:1624-1631.
- Campbell, D. R., N. M. Waser, M. V. Price, E. A. Lynch, e R. J. Mitchell. 1991. Components of phenotypic selection: pollen export and flower corolla width in *Ipomopsis aggregata*. *Evolution* 45:1458-1467.
- Campbell, D. R. 2009. Using phenotypic manipulations to study multivariate selection of floral trait associations. *Annals of Botany* 103:1557-1566.
- Carlson, J. E., e K. E. Holsinger. 2013. Direct and indirect selection on floral pigmentation by pollinators and seed predators in a color polymorphic South African shrub. *Oecologia* 171:905-919.
- Caruso, C. M., S. L. Scott, J. C. Wray, e C. A. Walsh. 2010. Pollinators, herbivores, and the maintenance of flower color variation: a case study with *Lobelia siphilitica*. *International Journal of Plant Science* 171:1020-1028.
- Castellanos, M. C., P. Wilson, e J. Thomson. 2004. 'Anti-bee' and 'pro-bird' changes during the evolution of hummingbird pollination in *Penstemon* flowers. *Journal of Evolutionary Biology* 17:876-885.
- Chittka, L., e N. M. Waser. 1997. Why red flowers are not invisible to bees. *Israel Journal of Plant Sciences* 45:169-183.
- Clegg, M. T., e M. L. Durbin. 2000. Flower color variation: a model for the experimental study of evolution. *Proceedings of the National Academy of Sciences of the United States of America* 97:7016-7023.
- Coberly, L., e M. Rausher. 2003. Analysis of a chalcone synthase mutant in *Ipomoea purpurea* reveals a novel function for flavonoids: amelioration of heat stress. *Molecular Ecology* 12:1113-1124.
- Darwin, C. 1862. On the Various Contrivances by which British and Foreign Orchids are Fertilised by Insects. Murray, London.
- Fenster, C. B., W. S. Armbruster, P. Wilson, M. R. Dudash, e D. James. 2004. Pollination syndromes and floral specialization. *Annual Review of Ecology, Evolution and Systematics* 35:375-403.
- Frey, F. M. 2004. Opposing natural selection from herbivores and pathogens may maintain floral-color variation in *Claytonia virginica* (Portulacaceae). *Evolution* 58:2426-2437.
- Frey, F. M. 2007. Phenotypic integration and the potential for independent color evolution in a polymorphic spring ephemeral. *American Journal of Botany* 94:437-444.
- Galen, C. 2000. High and dry: drought stress, sex-allocation trade-offs, and selection on flower size in the alpine wildflower *Polemonium viscosum* (Polemoniaceae). *The American Naturalist* 156:72-83.
- Gómez, J. M. 2003. Herbivory reduces the strength of pollinator-mediated selection in the mediterranean herb *Erysimum mediohispanicum*: consequences for plant specialization. *The American Naturalist* 162:242-256.

- Gómez, J. M., e R. Zamora. 2000. Spatial variation in the selective scenarios of *Hormathophylla spinosa* (Cruciferae). *The American Naturalist* 155:657-668.
- Gómez, J. M., J. Bosch, F. Perfectti, J. D. Fernández, M. Abdelaziz, e J. M. Camacho. 2008. Spatial variation in selection on corolla shape in a generalist plant is promoted by the preference patterns of its local pollinators. *Proceedings of the Royal Society B* 275:2241-2249.
- Grant, V. 1950. The flower constancy of bees. *The Botanical Review* 16:379-398.
- Harder, L. D., e S. D. Johnson. 2009. Darwin's beautiful contrivances: evolutionary and functional evidence for floral adaptation. *New Phytologist* 183:530-545.
- Herrera, C. M. 1993. Selection on floral morphology and environmental determinants of fecundity in a hawk moth-pollinated violet. *Ecological Monographs* 63: 251-275.
- Herrera, C. M. 2000. Measuring the effects of pollinators and herbivores: evidence for non-additivity in a perennial herb. *Ecology* 81:2170-2176.
- Herrera, C. M. 2001. Deconstructing a floral phenotype: do pollinators select for corolla integration in *Lavandula latifolia*? *Journal of Evolutionary Biology* 14:574-584.
- Herrera, C. M., M. Medrano, P. J. Rey, A. M. Sanchez-Lafuente, M. B. Garcia, J. Guitián, e A. J. Manzaneda. 2002. Interaction of pollinators and herbivores on plant fitness suggests a pathway for correlated evolution of mutualism- and antagonism-related traits. *Proceedings of the National Academy of Sciences of the United States of America* 99:16823-16828.
- Herrera, C. M., M. C. Castellanos, e M. Medrano. 2006. Geographical context of floral evolution: towards an improved research programme in floral diversification. Pages 278-294 *En* Harder, L. D., e S. C. H. Barret, eds. *Ecology and evolution of flowers*, Oxford University Press, Oxford, United Kingdom.
- Hodges, S. A., J. B. Whittall, M. Fulton, e J. Y. Yang. 2002. Genetics of floral traits influencing reproductive isolation between *Aquilegia formosa* and *Aquilegia pubescens*. *The American Naturalist* 159:51-60.
- Irwin, R. E., S. Y. Strauss, S. Storz, A. Emerson, e G. Guibert. 2003. The role of herbivores in the maintenance of a flower color polymorphism in wild radish. *Ecology* 84:1733-1743.
- Irwin, R. E., e S. Y. Strauss. 2005. Flower color microevolution in wild radish: evolutionary response to pollinator-mediated selection. *The American Naturalist* 165:225-237.
- Johnson, S., e K. Steiner. 1997. Long-tongued fly pollination and evolution of floral spur length in the *Disa draconis* complex (Orchidaceae). *Evolution* 51:45-53.
- Kolb, A., J. Ehrlén, e O. Eriksson. 2007. Ecological and evolutionary consequences of spatial and temporal variation in pre-dispersal seed predation. *Perspectives in Plant Ecology, Evolution and Systematics* 9:79-100.
- Kostic, M., S. Rajkovic, S. Drazic, e S. Stankovic. 2006. Bioagents of yellow gentian (*Gentiana lutea* L.). *Journal of Scientific Agricultural Research* 67:19-26.
- Lee, D. 2010. *Nature's palette: the science of plant color*. University of Chicago Press, Chicago, USA.
- Lev-Yadun, S., e K. S. Gould. 2009. Role of anthocyanins in plant defence. Pages 21-48 *En* Gould, K., K. Davies, e C. Winefield, eds. *Anthocyanins: biosynthesis, functions, and applications*, Springer, New York.
- Ma, J. F., P. R. Ryan, e E. Delhaize. 2001. Aluminium tolerance in plants and the complexing role of organic acids. *Trends in Plant Science* 6:273-278.
- Maad, J. 2000. Phenotypic selection in hawkmoth-pollinated *Platanthera bifolia*: targets and fitness surfaces. *Evolution* 54:112-123.

- Mascó, M., I. Noy-Meyr, e A. N. Sérsic. 2004. Geographic variation in flower color patterns within *Calceolaria uniflora* Lam. in Southern Patagonia. *Plant Systematics and Evolution* 244:77-91.
- Medrano, M., M. C. Castellanos, e C. M. Herrera. 2006. Comparative floral and vegetative differentiation between two European *Aquilegia* taxa along a narrow contact zone. *Plant Systematics and Evolution* 262:209-224.
- Mitchell-Olds, T., J. H. Willis, e D. B. Goldstein. 2007. Which evolutionary processes influence natural genetic variation for phenotypic traits? *Nature Reviews Genetics* 8:845-856.
- Obeso, J. R. 1992. Geographic distribution and community structure of bumblebees in the northern Iberian Peninsula. *Oecologia* 89:244-252.
- Parchman, T. L., e C. W. Benkman. 2002. Diversifying coevolution between crossbills and black spruce on Newfoundland. *Evolution* 56:1663-1672.
- Ploquin, E. F., J. M. Herrera, e J. R. Obeso. 2013. Bumblebee community homogenization after uphill shifts in montane areas of northern Spain. *Oecologia* 173:1649-1660.
- Price, T. D., A. Qvarnstrom, e D. E. Irwin. 2003. The role of phenotypic plasticity in driving genetic evolution. *Proceedings Biological Sciences / The Royal Society* 270:1433-1440.
- Rausher, M. D. 2008. Evolutionary transitions in floral color. *International Journal of Plant Science* 169:7-21.
- Rey, P. J., C. M. Herrera, J. Guitián, X. Cerdá, A. M. Sánchez-Lafuente, M. Medrano, e J. L. Garrido. 2006. The geographic mosaic in predispersal interactions and selection on *Helleborus foetidus* (Ranunculaceae). *Journal of Evolutionary Biology* 19:21-34.
- Robertson, J. L., e R. Wyatt. 1990. Evidence for pollination ecotypes in the yellow-fringed orchid, *Platanthera ciliaris*. *Evolution* 44:121-133.
- Rodríguez-Gironés, M. A., e L. Santamaría. 2004. Why are so many bird flowers red? *PLoS biology* 2:e350.
- Rossi, M. 2011. Taxonomy, phylogeny and reproductive ecology of *Gentiana lutea* L. PhD dissertation. Università degli studi di Bologna, Italia.
- Sánchez-Lafuente, A. M. 2002. Floral variation in the generalist perennial herb *Paeonia broteroi* (Paeoniaceae): differences between regions with different pollinators and herbivores. *American Journal of Botany* 89:1260-1269.
- Schemske, D. W., e H. D. Bradshaw Jr. 1999. Pollinator preference and the evolution of floral traits in monkeyflowers (*Mimulus*). *Proceedings of the National Academy of Sciences of the United States of America* 96:11910-11915.
- Stinchcombe, J. R., e M. D. Rausher. 2002. The evolution of tolerance to deer herbivory: modifications caused by the abundance of insect herbivores. *Proceedings Biological Sciences / The Royal Society* 269:1241-1246.
- Strauss, S. Y. 1997. Floral characters link herbivores, pollinators, and plant fitness. *Ecology* 78:1640-1645.
- Strauss, S. Y., e J. B. Whittall. 2006. Non-pollinator agents of selection on floral traits. Pages 120-138 *En Harder, L. D., e S. C. H. Barret, eds. Ecology and Evolution of Flowers*, Oxford University Press, New York.
- Strauss, S. Y., R. E. Irwin, e V. M. Lambrix. 2004. Optimal defence theory and flower petal colour predict variation in the secondary chemistry of wild radish. *Journal of Ecology* 92:132-141.

- Streisfeld, M. A., e J. R. Kohn. 2007. Environment and pollinator-mediated selection on parapatric floral races of *Mimulus aurantiacus*. *Journal of Evolutionary Biology* 20:122-132.
- Thompson, J. N. 1994. *The coevolutionary process*. University of Chicago Press, Chicago, USA.
- Thompson, J. N. 1997. Evaluating the dynamics of coevolution among geographically structured populations. *Ecology* 78:1619-1623.
- Whibley, A. C., N. B. Langlade, C. Andalo, A. I. Hanna, A. Bangham, C. Thébaud, e E. Coen. 2006. Evolutionary paths underlying flower color variation in *Antirrhinum*. *Science* 313:963-966.
- Yoshida, K., S. Kitahara, D. Ito, e T. Kondo. 2006. Ferric ions involved in the flower color development of the Himalayan blue poppy, *Meconopsis grandis*. *Phytochemistry* 67:992-998.
- Yoshida, K., Y. Toyama-Kato, K. Kameda, e T. Kondo. 2003. Sepal color variation of *Hydrangea macrophylla* and vacuolar pH measured with a proton-selective microelectrode. *Plant and Cell Physiology* 44:262-268.
- Zangerl, A., e M. Berenbaum. 2003. Phenotype matching in wild parsnip and parsnip webworms: causes and consequences. *Evolution* 57:806-815.
- Zhu, C., S. Yamamura, H. Koiwa, M. Nishihara, e G. Sandmann. 2002. cDNA cloning and expression of carotenogenic genes during flower development in *Gentiana lutea*. *Plant Molecular Biology* 48:277-285.
- Zhu, C., S. Yamamura, M. Nishihara, H. Koiwa, e G. Sandmann. 2003. cDNAs for the synthesis of cyclic carotenoids in petals of *Gentiana lutea* and their regulation during flower development. *Biochimica et Biophysica Acta (BBA)-Gene Structure and Expression* 1625:305-308.





2. PLANTA E ÁREA DE ESTUDO



2.1 HISTORIA NATURAL DA PLANTA

Gentiana lutea L., pertence ó xénero *Gentiana* da familia *Gentianaceae*; o xénero comprende arredor de 361 especies que medran na súa maioría en zonas temperadas e alpinas de Asia. A distribución de *G. lutea* abarca dende os Montes Urais ata a Península Ibérica, sempre polas cordilleiras, nun rango altitudinal que abarca dende os 900 ata os 2400 m por enriba do nivel do mar (Kohlein 1991). A distribución da xanzá na Cordilleira Cantábrica responde ó patrón típico das plantas de montaña do norte da Península Ibérica cun patrón de distribución disxunta (Vargas 2003, Kropf et al. 2008, Schneeweiss e Schönswetter 2010).

A xanzá é unha especie xeófita, posúe un rizoma que sobrevive durante anos. Na área xeográfica do norte de España, nos inicios do verán (xuño), desenvólvense unhas follas oblongas con nervación paralela formando unha ou varias rosetas. Xa co verán máis avanzado, en xullo, as plantas poden desenrolar (non o fan tódolos anos) un escapo, inda que raramente poden saír dous e ata tres talos dunha mesma roseta, de arredor 1.40 m de altura, que porta as flores. Este escapo está dividido en nós (de 7 a 9) separados polos entrenós; os nós superiores (3-5) desenrolan enriba de cada bráctea un grupo de flores e cada nó florido porta entre 15-30 flores. O escapo remata nunha flor na cima e desenrola en torno a 80 flores (Struwe e Albert 2002). As flores son actinomorfas, con pétalos libres (4-8), estames libres (4-8), un estilo que remata en dous estigmas recurvados e un ovario constituído por dous carpelos. En cada froito se desenrolan arredor de 80 sementes aladas, de pequeno tamaño (3 mm), aplanadas e con forma circular-elíptica. Ó final do verán os froitos sécanse e abren (Struwe e Albert 2002).

Gentiana lutea é unha especie xeneralista, insectos pertencentes a catro ordes diferentes (Himenóptera, Díptera, Coleóptera e Lepidóptera) polinizan esta especie (Rossi 2011). Os polinizadores máis frecuentes son abellons (xénero *Bombus*, orde Himenóptera).

A xanzá sofre diferentes tipos de herbivoría tanto nas partes aéreas vexetativas e florais, coma nos froitos e sementes (Kostic et al. 2006). Na área de estudo, atopamos diversos axentes responsables desta herbivoría, dende as larvas de Lepidópteros que se alimentan das sementes (observacións persoais), ata insectos que perforan os talos provocando a caída enteira destes, ou mamíferos (vacas, cabalos, corzos) que consomen as flores durante a floración e os froitos durante a frutificación temperá (observacións propias). A dispersión das sementes é anemócora: unha vez os froitos están secos e abertos, o vento move os talos e as sementes saen dos froitos portadas lonxe da planta nai polo vento antes de caer (Struwe e Albert 2002).

Esta especie posúe propiedades medicinais. O rizoma contén compostos amargos, razón pola cal se utilizou para elaborar remedios dixestivos e licores que aumentan o apetito coma o vermú (Tonutti e Liddle 2010). A colleita dos rizomas foi unha actividade intensa en casi toda a súa área de distribución no norte de España, mingando as poboacións ata o punto no que, hoxe en día, a xanzá é unha especie protexida, catalogada como vulnerable pola Directiva de Hábitats Europea; por este motivo, a súa colleita está controlada e limitada (Herrero e Martín-Lobera 2012).



Figura 2.1. *Gentiana lutea* var. *lutea* (esquerda; imaxes da poboación de Ventana) e *G. l.* var. *aurantiaca* (dereita; imaxe superior da poboación de San Mamede e inferior da poboación de Ancares). Fotografías de Pablo Guitián.

Os hábitats característicos nos que medra son pasteiros de montaña, sobre substrato básico maiormente, inda que na Península Ibérica tanto aparece sobre solos ácidos coma básicos. As plantas posúen flores tipicamente amarelas (*Gentiana lutea* var. *lutea*; Fig. 2.1), agás no extremo occidental da súa distribución (oeste da Península Ibérica) onde atopamos poboacións que posúen plantas con flores de cor laranxa (*Gentiana lutea* var. *aurantiaca*; Fig. 2.1; Renobales 2012). Tanto a superficie ocupada polas poboacións, como a densidade de individuos varía: atopamos poboacións que abarcan dende 12 hectáreas ata menos de 300 m²; e densidades que flutúan entre os 30 individuos floridos por 100 m² até os 450 individuos con escapos en flor por 100 m² (observacións propias).

2.2 ÁREA DE ESTUDO

A área de estudo comprende o extremo occidental da Cordilleira Cantábrica, (suroeste de Europa), dende San Mamede, Ourense (7° 30' W, límite occidental) até San Glorio, León (4° 45' W, límite oriental da área de estudo); abarcando un total de 230 km (Fig. 2.2). As doce poboacións estudadas sitúanse entre os 900 e os 1800 m de altitude por enriba do nivel do mar, sobre substratos ácidos ou básicos. A maioría se

localizan sobre pasteiros de montaña, prados de sega, comunidades de megaforbias, breixeiras, piornais, claros de faiais e piñeirais. As temperaturas das localidades onde se sitúan as plantas estudadas varían entre os 0 °C de media invernal (media de decembro, xaneiro e febreiro) ata os 14 °C de media estival (media de xuño, xullo e agosto). A precipitación ascende a 175 mm como máxima durante o inverno (media de decembro, xaneiro e febreiro) e descende ós seus niveis inferiores durante o verán con 55 mm (media de xuño, xullo e agosto); a media anual de precipitación atópase en 1300 mm (datos que proveñen dun período de 15-50 anos, entre 1951-1999; Ninyerola et al. 2005).

Os contrastes bioclimáticos máis significativos do territorio son esencialmente dous: a diminución das precipitacións estivais de oriente a occidente da Cordilleira Cantábrica (por exemplo de Picos de Europa a Courel-Ancas), e o incremento da continentalidade na vertente meridional da liña divisoria da Cordilleira (por exemplo de Somiedo a Babia; Rivas-Martínez et al. 1984). Estas características son responsables da distribución de moitas especies e comunidades vexetais no territorio.

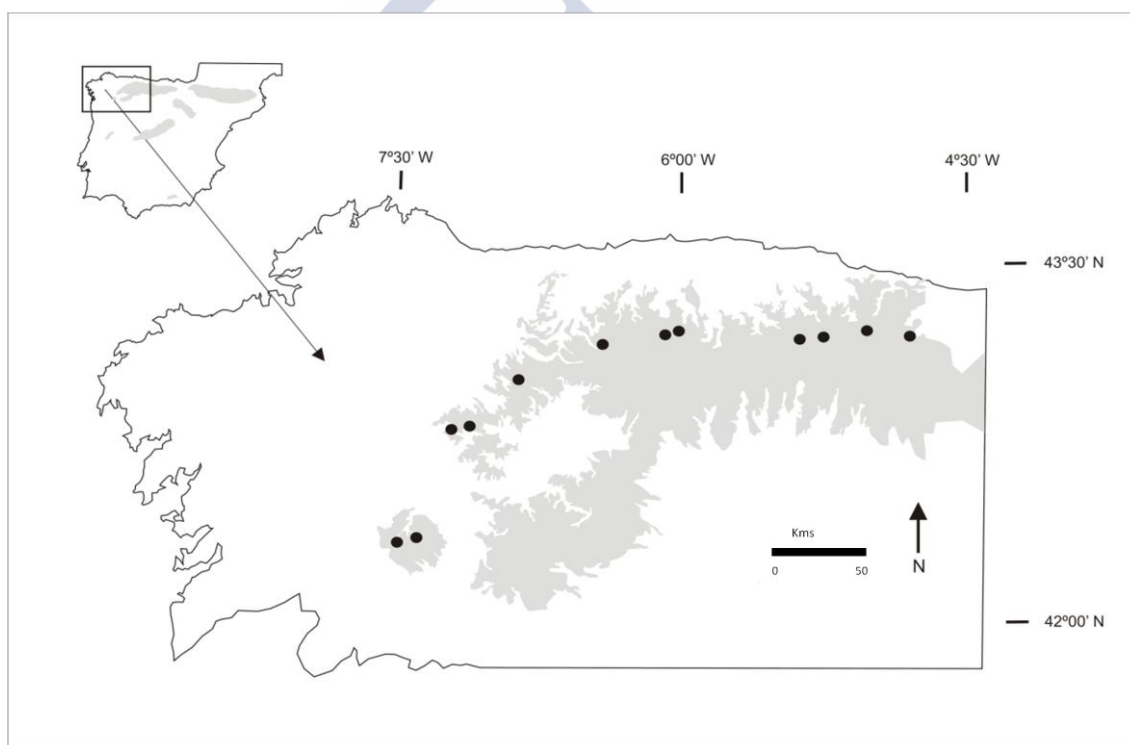


Figura 2.2. Distribución xeográfica de *Gentiana lutea* na Península Ibérica (área sombreada) e localización das poboacións estudadas (puntos).

2.3 A COR FLORAL

A cor das flores é o carácter central deste traballo polo tanto son imprescindibles medidas precisas que reflectan a variación cuantitativa da cor. Obtivemos os espectros da cor dos pétalos mediante un espectrómetro (USB2000+; Ocean Optics, Inc., Dunedin, FL). Cada pétalo foi medido inmediatamente tras ser recollido e as medidas se tomaron na cara adaxial no medio do pétalo. As condicións técnicas das medidas foron as seguintes: antes do comezo de cada sesión de medida (pola mañá e pola tarde), calibramos o espectrómetro utilizando un branco estándar, a continuación procedimos a

medir, controlando que a única fonte de luz proviñese dunha lámpada de deuterio e tungsteno (DT-MINI-2-GS) e mantendo un ángulo de incidencia fixo en 45°. A cor floral de cada planta resultou da media dos espectros de 3 medidas de cada un dos 3 pétalos de entre 3 e 10 flores por planta (entre 27 e 90 medidas de cor por planta) dependendo dos obxectivos de cada capítulo. O programa utilizado para procesar os espectros de reflectancia foi SpectraSuite (Ocean Optics, Inc., Dunedin, FL, USA).

A cor é un atributo subxectivo, é unha percepción que depende do sistema visual do observador (e doutros aspectos coma a luz ambiental, o que depende á súa vez de factores atmosféricos; Endler 1993). Para describir a cor tendo en conta os axentes de selección, deberíamos coñecer o sistema visual das diferentes especies, mais non contamos con esta información. O que parece común e xeral nos insectos é a detección da luz ultravioleta (Briscoe e Chittka 2001), así que sería axeitado describir a cor das flores considerando tamén esta parte do espectro da luz. Sen embargo, neste traballo procuramos describir a selección natural sobre a cor das flores nunha especie que posúe variación de laranxa a amarelo. Se non existen diferenzas na luz UV entre ambas cores (percibidas por ollo humano) e entre poboacións, podemos concluír que os polinizadores non diferenzan as plantas laranxas e amarelas pola luz UV e que non existe adaptación local da emisión UV. Por tanto, podemos centrarnos no rango visible do espectro para describir a selección natural sobre a cor das flores.

Estudamos o rango UV en 100 plantas pertencentes a poboacións que posuían ou tódalas plantas con flores amarelas, ou tódalas plantas con flores laranxas, para así facilitar a distinción entre ambas cores. Seleccionamos ó azar 50 plantas laranxas pertencentes a cinco poboacións e 50 amarelas pertencentes a outras catro poboacións. Para cada planta utilizamos o valor da reflectancia entre os 300 nm e os 400 nm (rango UV), dividido en 11 intervalos de 10 nm; así, cada planta posúe a reflectancia do rango UV caracterizado por once variables. Mediante unha análise de compoñentes principais (PCA), reduxemos as once variables. O primeiro factor explicou o 75% da variabilidade (valor eigen = 8.26); por tanto, consideramos que é válido para describir a variación da cor no rango UV. Mediante unha análise de varianza aniñado, comprobamos se as poboacións e as plantas con flores de diferentes cores, difiren no rango ultravioleta. A variable resposta foi o primeiro factor obtido da PCA e as variables independentes foron a cor, con dúas categorías, laranxa e amarelo, e a poboación aniñada na cor. O resultado mostra que non hai diferenzas na emisión de luz ultravioleta nin entre poboacións nin entre plantas con flores de diferentes cores (laranxa e amarelo; $F_{7,91} = 1.77$, $P = 0.10$; $F_{1,91} = 1.54$, $P = 0.22$; respectivamente para poboación e cor; Fig. 2.3).

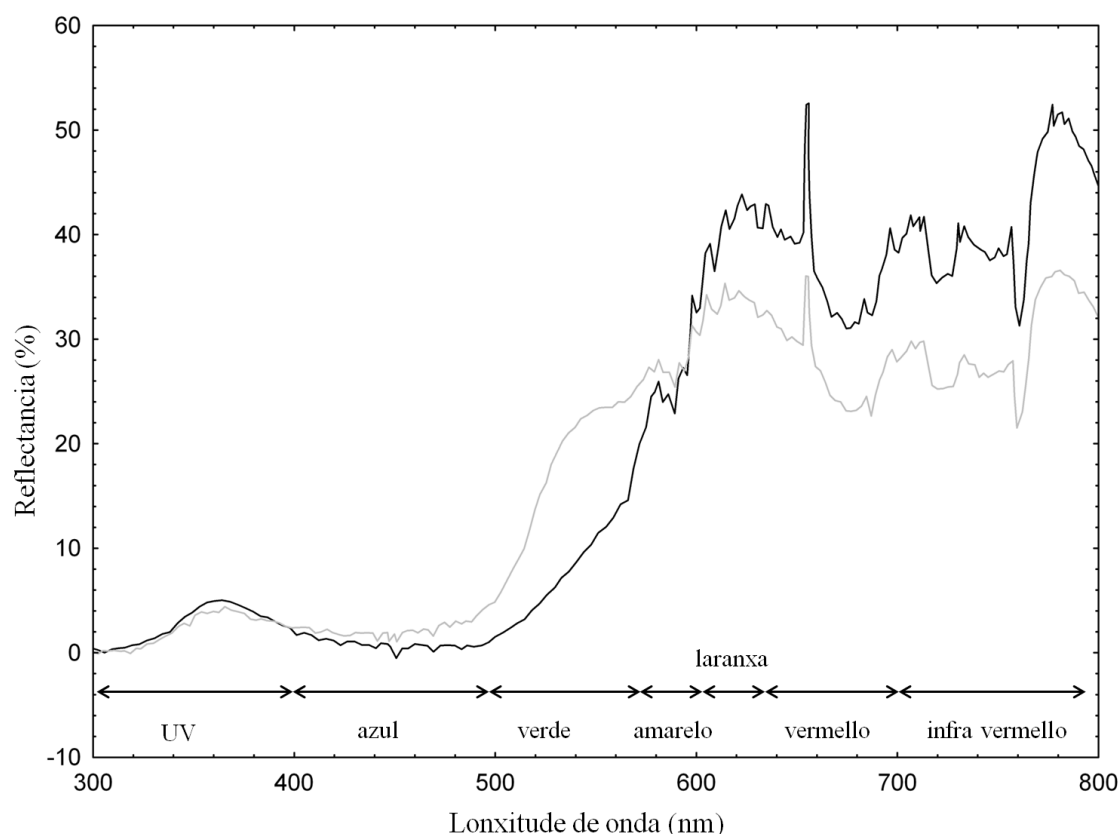


Figura 2.3. Media do espectro de reflectancia para flores amarelas (liña gris) e flores laranxas (liña negra).

Xa que non hai diferenzas nin entre poboacións nin entre as cores laranxa e amarela no espectro ultravioleta, para describir a cor utilizamos o sistema de colorimetría CIELab, baseado no rango visible do espectro; é dicir, na percepción humana. Neste sistema, o espectro de reflectancia no rango visible se transforma en tres variables (L , a , b) que en conxunto describen a cor. Consideramos que este sistema é axeitado para describir a variabilidade da cor na xanzá (laranxa-amarela) porque unha das variables (a) recolle a variación no vermello e outra das variables (b) recolle a variación no amarelo. A terceira variable (L) informa sobre o brillo.

Os espectros de reflectancia, dende os 380 nm ata os 780 nm, a intervalos de 5 nm; foron transformados nas variables de cor do sistema de colorimetría CIELab (CIE 2004). Neste sistema L varía de 0 (negro) a 100 (branco); a varía de -110 a 110, o que se traduce a unha variación na cor de verde a vermello; e b varía de -110 a 110, de tal forma que valores negativos indican azul e positivos amarelo. En conxunto, as tres variables definen un espazo tridimensional, no que a cor varía como un continuo; é o diagrama de cromaticidade CIELab. Os parámetros empregados foron o observador estándar CIE 1964 (campo visual de 10°) e o iluminante A estándar.

2.4 O SISTEMA REPRODUTIVO

Moitas das plantas con flor dependen dos polinizadores para reproducirse con éxito. Esta relación planta-polinizador promove a evolución das especies implicadas na interacción e por iso hai numerosos estudos centrados na evolución de rasgos florais por medio dos polinizadores. Para xustificar o efecto dos polinizadores, primeiro, hai que demostrar que os polinizadores teñen un efecto no éxito reprodutivo das plantas.

O éxito reprodutivo debe cuantificarse como o número de descendentes fértiles que produce un individuo ó longo da súa vida. Cuantificar o éxito reprodutivo nestes termos, en especies de vida longa, é inviable de modo que se considera que a produción de sementes é unha boa estima (ver por exemplo a revisión de Kingsolver et al. 2001). Se queremos medir o éxito reprodutivo en etapas posteriores á produción de sementes, podemos utilizar a viabilidade das sementes e a taxa de xerminación. Nos experimentos descritos a continuación, imos utilizar estas tres medidas do éxito reprodutivo: o número de sementes, a taxa de sementes viables (viabilidade) e a taxa de xerminación.

No contexto desta investigación resulta necesario probar que as plantas dependen dos polinizadores para reproducirse e ademais pode ser interesante coñecer se existe algún grao de incompatibilidade entre plantas de diferentes cores. Por iso nos plantexamos as seguintes preguntas: ¿hai diferenzas no éxito reprodutivo—número de sementes, viabilidade e xerminación—entre os froitos que proveñen dunha autofertilización e os que proveñen dun cruzamento entre plantas diferentes? ¿Hai diferenzas no éxito reprodutivo segundo a cor floral das plantas donadoras de pole?

Para resolver as cuestións previas, levamos a cabo manipulacións experimentais na poboación de Torrestío en xullo de 2011. Seleccionamos aleatoriamente 25 plantas laranxas e 25 plantas amarelas, e en cada planta escollimos aleatoriamente cinco flores, destinadas a tratamentos diferentes. Tódalas flores, agás as flores do grupo control, foron embolsadas con tul antes de producirse a apertura floral e ata a formación do froito para evitar o contacto con polinizadores.

Os tratamentos foron os seguintes: (1) grupo control (cruzamento espontáneo, Ce), as flores non se manipularon; (2) autopolinización espontánea (Ae), non aplicamos pole; (3) autopolinización facilitada (Af), aplicamos manualmente pole das propias anteras; (4) cruzamento entre plantas da mesma cor floral (Cf), aplicamos manualmente pole doutras plantas da mesma cor e emasculamos as flores cortando os estames antes da liberación de pole para evitar a entrada de pole propio; (5) cruzamento entre plantas de distinta cor floral (Cc), aplicamos manualmente pole doutras plantas de diferente cor e tratamos os estames coma no caso anterior.

O número de plantas cos cinco tratamentos se reduxo a 26, debido a que entre a polinización manual e a recollida dos froitos, algunhas bolsas se abriron e algunhas plantas foron danadas. Polo tanto, únicamente se recolleron os 130 froitos destas plantas. Os froitos maduros foron recollidos antes da súa apertura, coidando que as sementes estiveran totalmente formadas. En cada froito contamos o número de sementes máis o número de óvulos non desenvolvidos; da suma de óvulos non fertilizados máis as sementes, obtivemos o número total de óvulos. Debido a que o número total de óvulos non variou entre os tratamentos (datos non mostrados), en lugar de calcular a produción de sementes relativa ós óvulos en cada froito utilizamos directamente o número absoluto de sementes producidas en cada froito.

Medimos tamén a taxa de xerminación e viabilidade das sementes. De cada froito escollimos aleatoriamente 20 sementes e no caso de que os froitos posúiran menos de 20 sementes, collímolos todas. As sementes dun mesmo tratamento xuntáronse e distribuíronse sobre papel de filtro en 10 placas petri, con 50 sementes por placa. Debido a que a cantidade de sementes era baixa nos tratamentos de autogamia, estes dous tratamentos foron agrupados; en calquera caso, cada placa de autogamia posuía como mínimo 30 sementes. En total contamos con 40 placas petri e 1800 sementes (10 por cada tratamento, catro tratamentos, 30 placas con 50 sementes, 10 placas con 30 sementes). A xerminación foi inducida con ácido giberélico 100 mg/L baixo 24 h de

escuridade e temperatura constante de 23 °C. O estado de xerminación e humectación das placas foron controlados en días alternos; o papel de filtro foi mudado cada 2-3 días para diminuír a contaminación por fungos. As sementes con infección por fungos desbotáronse. As sementes que posuían ó menos 2 mm de radícula foron consideradas xerminadas. A taxa de xerminación dunha placa é a proporción de sementes non infectadas por fungos que xerminaron. Controlamos a taxa de xerminación durante un período de 45 días.

Tras os 45 días testamos a viabilidade das sementes. Este test consistiu en aplastar as sementes ca punta das pinzas. As sementes blandas e escuras foron consideradas inviáveis e as duras e de cor clara viáveis (metodoloxía descrita e validada por Sawma e Mohler 2002, Hesse 2007). Estas sementes mais as sementes xa xerminadas foi considerado o número total de sementes viáveis.

Utilizamos un modelo liñal xeneralizado mixto pra analizar as diferenzas no número de sementes segundo os tratamentos e a cor das flores. Os factores fixos foron: o tratamento (cinco categorías), a cor floral, a interacción entre cor e tratamento e o factor aleatorio foi a planta. Para probar a existencia de diferenzas na viabilidade e xerminación en función do tratamento de polinización, utilizamos un modelo liñal xeneralizado para cada variable. O factor foi o tratamento (con catro categorías, xa que as sementes dos tratamentos de autopolinización espontánea e facilitada se xuntaron para ter unha mostra suficiente) e introduxemos como covariable a taxa de infección por fungos (a pesar de que as sementes infectadas foron eliminadas, o número de sementes infectadas pode indicar o grao de contaminación de toda a placa, o cal pode afectar á xerminación). Nótese que non tivemos mostra suficiente para separar os tratamentos entre cores e por tanto non puidemos probar se a taxa de xerminación variaba entre cores. A distribución do erro e a función de enlace se seleccionaron para minimizar o AICC: o número de sementes respondeu a unha distribución poisson e unha función de enlace logarítmica; tanto a viabilidade coma a xerminación foron axustadas a unha distribución normal e función de enlace identidade. Realizamos tamén tests post-hoc de comparación por pares, no caso de que un factor fose estatisticamente significativo (P valor < 0.05). O programa estatístico empregado foi o SPSS, version 20 para windows (IBM SPSS Statistics 20; IBM Corp., Somers, NY).

Os resultados mostran que os froitos baixo os tratamentos de autopolinización (espontánea e facilitada) produxeron un número menor de sementes (sete sementes de media por froito) que os froitos baixo os tratamentos de polinización cruzada (Cf: 98, Cc: 98, Ce: 81; media de sementes por froito). Destaca que, os froitos dos cruzamentos controlados (Cf e Cc), produxeron un número similar de sementes, sen importar a cor do donador de pole. Ademais, non atopamos diferenzas na resposta ós tratamentos entre plantas de flores laranxas e plantas de flores amarelas (Táboa 2.1, Fig. 2.4a).

Táboa 2.1. Efecto dos distintos tratamentos de polinización no éxito reprodutivo (número de sementes, viabilidade e xerminación). No caso do número de sementes, utilizamos un modelo liñal xeneralizado mixto e os estatísticos son F e Z para factores fixos e aleatorios respectivamente. Nos casos da viabilidade e xerminación utilizamos modelos liñais xeneralizados e o estatístico é un Chi-cadrado. Destacamos en negra os factores estatisticamente significativos ($P < 0.05$).

Variable dependente	N	Factor	F	Z	χ^2	g.l.	P
Número de sementes	130	Tratamento	31.615			4;27	0.000
		Cor	0.000			1;53	0.985
		Tratamento*cor	1.780			4;27	0.162
		Planta		2.383			0.017
% Viabilidade	40	Tratamento			11.301	3;35	0.010
		Infección por fungos			11.258	1;35	0.001
% Xerminación	40	Tratamento			22.568	3;35	0.000
		Infección por fungos			20.750	1;35	0.000



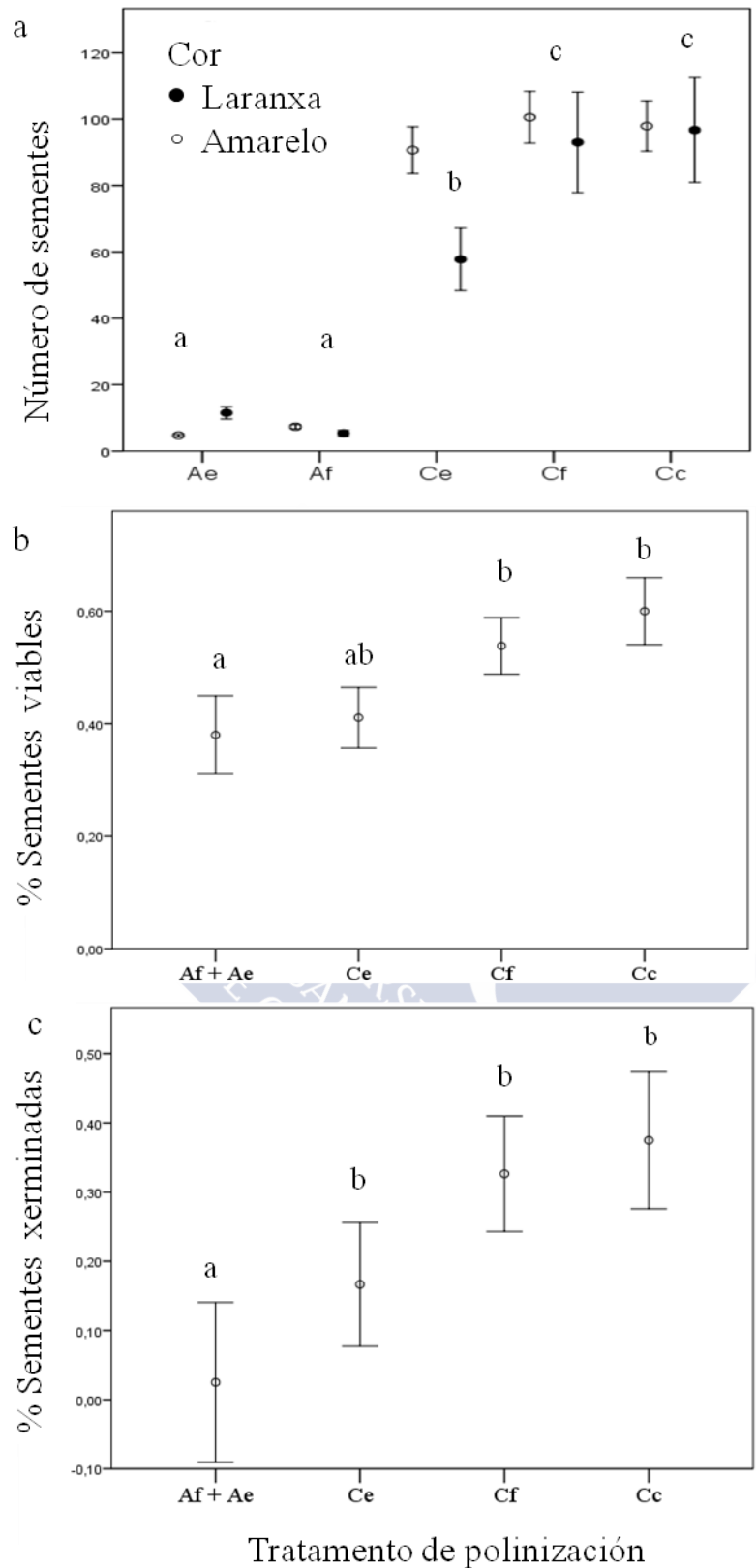


Figura 2.4. Éxito reprodutivo en función dos diferentes tratamentos de polinización. a) Número de sementes, b) Viabilidade, c) Xerminación. Os tratamentos de polinización foron os seguintes: autopolinización espontánea (Ae) e autopolinización facilitada (Af); cruzamento espontáneo (Ce), cruzamento facilitado entre plantas de cor floral similar (Cf) e cruzamento entre plantas de cor floral distinta (Cc). As diferenzas entre os tratamentos se denotan con letras diferentes. Móstranse as medias e o intervalo de confianza do 95%.

A viabilidade foi maior no caso das sementes do cruzamento facilitado, comparado cos tratamentos de autopolinización; ademais a viabilidade das sementes do grupo control (cruzamento espontáneo) foi similar a tódolos tratamentos restantes (Táboa 2.1, Fig. 2.4b). A xerminación das sementes que proveñen de polinización cruzada (Ce, Cf e Cc) foi maior que a xerminación de sementes que proveñen de autopolinización (Ae e Af; Táboa 2.1, Fig. 2.4c).

Os nosos resultados máis relevantes poñen de manifesto que os polinizadores afectan ó éxito reprodutivo, de tal modo que aumentan tanto o número de sementes, como a viabilidade e a taxa de xerminación de *Gentiana lutea*. Inda que os froitos autopolinizados produzan sementes, producen moitas menos sementes (Fig. 2.4a) e estas sementes son de peor calidade xa que a proporción de sementes viables e xerminadas é menor que no caso dos cruzamentos controlados (Fig. 2.4b e 2.4c). Por tanto, podemos considerar que esta especie está cercana á autoincompatibilidade absoluta e que depende dos polinizadores para reproducirse con éxito.

Resulta interesante o feito de que a proporción de sementes xerminadas procedentes do tratamento de cruzamento espontáneo é intermedia entre a autopolinización e a polinización facilitada. Este resultado suxire que os polinizadores portan, en condicións naturais, tanto polen da propia planta como de plantas diferentes, de tal modo que a calidade das sementes é menor, con respecto ó tratamento de polinización facilitada, no que todo o pole procede de plantas diferentes. En consecuencia, aqueles polinizadores que posúan maior mobilidade entre plantas e menor mobilidade entre flores do mesmo individuo, poderían ser máis beneficiosos para as plantas.

2.5 CONSIDERACIÓNS SOBRE O SOLO

A cor das flores pode variar en relación a certos factores abióticos coma por exemplo son os factores edáficos. Características do solo coma o pH e a concentración de aluminio poden afectar a cor das flores (Ma et al. 2001, Yoshida et al. 2003, Yoshida et al. 2006). Na Península Ibérica, o sustrato varía lonxitudinalmente de oriente a occidente, de tal modo que a Cordilleira Cantábrica se pode dividir en dúas áreas: o leste con solos básicos, e o oeste con solos ácidos (Moreno-Saiz et al. 2013). A distribución de *G. lutea* var. *aurantiaca*, variedade caracterizada por posuír as flores laranxas, abarca o oeste da Cordilleira Cantábrica (Renobales 2012), polo que o patrón de variación na cor das flores podería encaixar con esta ordenación según criterios edáficos.

Para comprobar se a cor das flores de *Gentiana lutea* varía en función do pH do substrato, en xullo de 2012, recollimos mostras de solo en once poboacións nas que medran plantas con flores amarelas e plantas con flores laranxas. En cada poboación, recollimos unha mostra do solo situado debaixo dunha planta con flores amarelas e baixo unha planta con flores laranxas. As mostras de solo secáronse ó aire e tamizáronse (tamaño da malla, 2 mm). Mesturouse o solo con auga, nunha proporción 1:2.5 para obter tanto o pH, coma o pH en KCl 0.1 M (potencial de acidez). Determinamos o pH cun pHmetro (Crison micropH 2001), tras un tempo de espera de 10 minutos para o pH en auga e de 2 horas para o pH en KCl (Gutián e Carballas 1976). As análises leváronse a cabo no laboratorio de Edafoloxía e Química Agrícola da Universidade de Santiago de Compostela.

Estudamos a relación entre o pH e a cor da flores mediante un modelo liñal xeneralizado, considerando a cor das flores como a variable resposta (distribución binomial e función de enlace logit) e tanto o pH coma o potencial de acidez (pH en

KCl) como variáveis independentes. Nenhuma destas variáveis resulta ser estatisticamente significativa (Chi-cadrado = 0.291, g.l. = 1, P = 0.590; Fig. 2.5; Chi-cadrado = 0.428, g.l. = 1, P = 0.513; respectivamente para o pH e o pH em KCl). Os resultados deste estudo indicam que a variación na cor das flores em *Gentiana lutea* non depende do pH do solo.

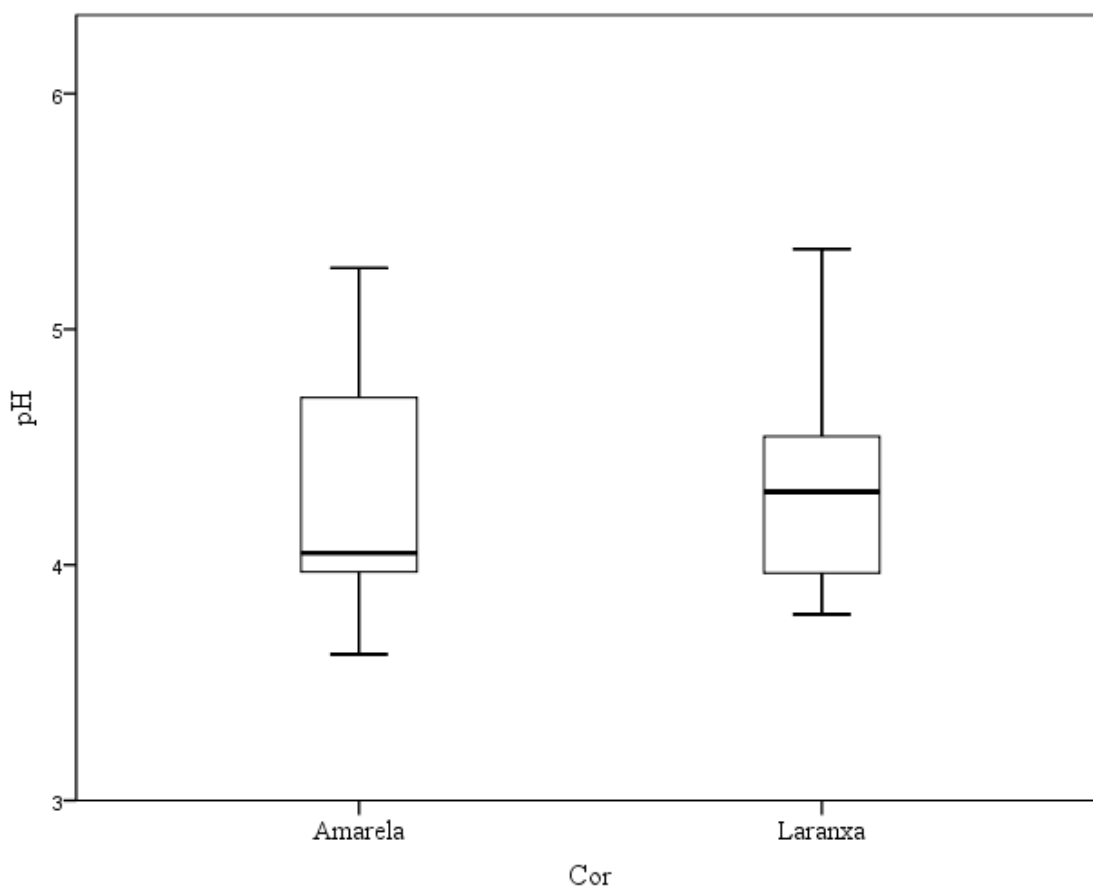
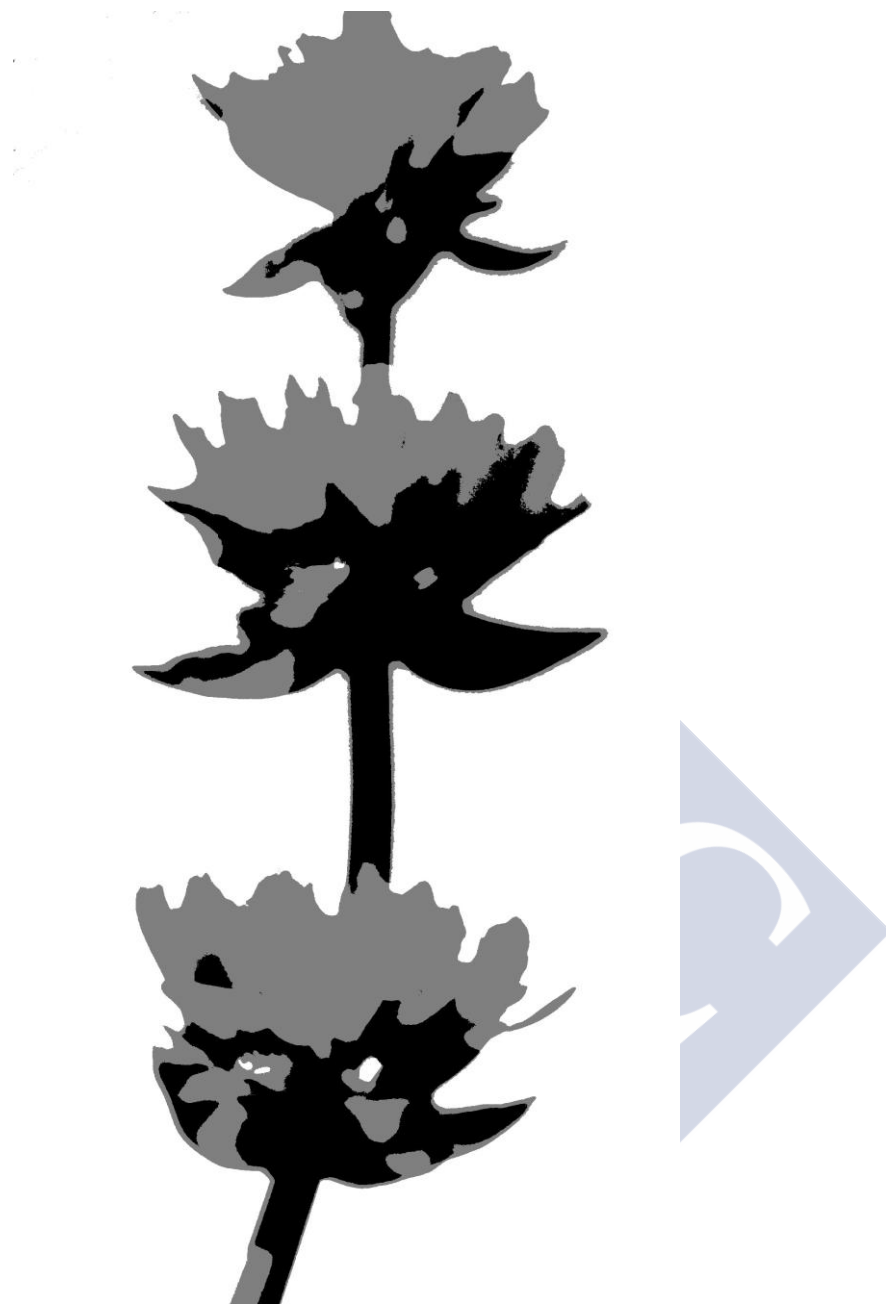


Figura 2.5. Diagrama de caixa mostrando a variación do pH do solo baixo plantas con flores amarelas ou laranxas. A liña representa a mediana, as caixas os cuartís e os bigotes os valores mínimo e máximo.

2.6 REFERENCIAS

- Briscoe, A. D., e L. Chittka. 2001. The evolution of color vision in insects. *Annual Review of Entomology* 46:471-510.
- CIE, P. 2004. 15: 2004. Colorimetry. Vienna, Austria: CIE Central Bureau.
- Endler, J. A. 1993. The color of light in forests and its implications. *Ecological Monographs* 63:2-27.
- Gutián F., e T. Carballas. 1976. Técnicas de análisis de suelos. Pico Sacro, Santiago de Compostela, Spain.
- Herrero, B., e C. Martín-Lobera. 2012. Threatened medicinal plants in Castilla y León (Spain). *Global Advanced Research Journal of Medicinal Plants* 1:1-6.
- Hesse, E., M. Rees, e H. Müller-Schärer. 2007. Seed bank persistence of clonal weeds in contrasting habitats: implications for control. *Plant Ecology* 190:233-243.
- IBM Corp. Versión 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp.

- Kingsolver, J. G., H. E. Hoekstra, J. M. Hoekstra, D. Berrigan, S. N. Vignieri, C. E. Hill, A. Hoang, P. Gibert, e P. Beerli. 2001. The strength of phenotypic selection in natural populations. *The American Naturalist* 157:245-261.
- Kohlein, F. 1991. *Gentians*. Timber Press Inc, Portland, Oregon, USA.
- Kostic, M., S. Rajkovic, S. Drazic, e S. Stankovic. 2006. Bioagents of yellow gentian (*Gentiana lutea* L.). *Journal of Scientific Agricultural Research* 67:19-26.
- Kropf, M., H. P. Comes, e J. W. Kadereit. 2008. Causes of the genetic architecture of south-west European high mountain disjuncts. *Plant Ecology and Diversity* 1:217-228.
- Ma, J. F., P. R. Ryan, e E. Delhaize. 2001. Aluminium tolerance in plants and the complexing role of organic acids. *Trends in Plant Science* 6:273-278.
- Ninyerola, M., J. M. Roure, e X. P. Fernández. 2005. Atlas climático digital de la Península Ibérica: metodología y aplicaciones en bioclimatología y geobotánica. Universidad Autónoma de Barcelona, Bellaterra, Barcelona, Spain.
- Rivas-Martínez, S., T. E. Díaz, J. A. F. Prieto, J. Loidi and A. Penas. 1984. *La Vegetación de la Alta Montaña Cantábrica: Los Picos de Europa*. Ediciones Leonesas. León.
- Renobales, S. G. 2012. *Gentiana*. En Castroviejo, S., S. Talavera, C. Andrés, M. Arista, M. P. Fernández-Piedra, M. J. Gallego, P. L. Ortiz, C. Romero-Zarco, F. J. Salgueiro, S. Silvestre, and A. Quintanar, eds. *Flora Ibérica Vol. XI. Plantas vasculares de la Península Ibérica e Islas Baleares, Gentianaceae-Boraginaceae*, Real Jardín Botánico, CSIC, Madrid, Spain.
- Rossi, M. 2011. Taxonomy, phylogeny and reproductive ecology of *Gentiana lutea* L. PhD dissertation. Università degli studi di Bologna, Italia.
- Sawma, J. T., e C. L. Mohler. 2002. Evaluating seed viability by an unimbibed seed crush test in comparison with the tetrazolium test 1. *Weed Technology* 16:781-786.
- Schneeweiss, G., e P. Schönswetter. 2010. The wide but disjunct range of the European mountain plant *Androsace lactea* L. (Primulaceae) reflects Late Pleistocene range fragmentation and post-glacial distributional stasis. *Journal of Biogeography* 37:2016-2025.
- Struwe, L., e V. A. Albert. 2002. *Gentianaceae: Systematics and natural history*. Cambridge University Press, Cambridge, UK.
- Tonutti, I., e P. Liddle. 2010. Aromatic plants in alcoholic beverages. A review. *Flavour and Fragrance Journal* 25:341-350.
- Vargas, P. 2003. Molecular evidence for multiple diversification patterns of alpine plants in Mediterranean Europe. *Taxon* 52:463-476.
- Yoshida, K., S. Kitahara, D. Ito, e T. Kondo. 2006. Ferric ions involved in the flower color development of the Himalayan blue poppy *Meconopsis grandis*. *Phytochemistry* 67:992-998.
- Yoshida, K., Y. Toyama-Kato, K. Kameda, e T. Kondo. 2003. Sepal color variation of *Hydrangea macrophylla* and vacuolar pH measured with a proton-selective microelectrode. *Plant and Cell Physiology* 44:262-268.
- Zhu, C., S. Yamamura, H. Koiwa, M. Nishihara, e G. Sandmann. 2002. cDNA cloning and expression of carotenogenic genes during flower development in *Gentiana lutea*. *Plant Molecular Biology* 48:277-285.
- Zhu, C., S. Yamamura, M. Nishihara, H. Koiwa, e G. Sandmann. 2003. cDNAs for the synthesis of cyclic carotenoids in petals of *Gentiana lutea* and their regulation during flower development. *Biochimica et Biophysica Acta (BBA)-Gene Structure and Expression* 1625:305-308.



3.FLORAL AND VEGETATIVE
VARIATION AMONG
Gentiana lutea L. POPULATIONS



3.1 ABSTRACT

Floral and vegetative variation among plant populations may result from the spatial variation of selective agents. Spatial distribution of phenotypic variation within a species—in particular, the comparison of floral versus vegetative variation—informs on the ecological processes driving this variation. Variation in floral traits is, mainly, attributed to variation in the pollinator fauna, while variation in vegetative traits is usually linked to abiotic factors or herbivores. Neither the spatial variation of floral or vegetative traits nor the geographical distribution of this variation was previously described in *Gentiana lutea*. We investigated the spatial variation in floral and vegetative traits and its relationship with abiotic factors, among 12 populations of *G. lutea* located across the western end of its distribution. Populations differed in floral and vegetative traits and these differences were not related to abiotic factors. Flower color and petal width were the most variable traits among populations. Variation of floral and vegetative traits, were geographically structured although our results indicate that floral variation, in particular flower color, drags phenotypic variation in *G. lutea*.

3.2 INTRODUCTION

Floral and vegetative phenotypic variation among populations of a plant species may follow different patterns (Armbruster et al. 1999). As a first step to understand the microevolutionary process, we may describe the spatial variation of phenotypes (Gould and Johnston, 1972). Environmental (biotic and abiotic) heterogeneity may cause different selective pressures resulting in phenotypic variation among populations (Linhart and Grant 1996, Schluter 2009). Selective forces exerting pressure on flower traits usually differ from those on vegetative attributes; thus, comparing the variation of vegetative and floral traits provides information about the ecological adaptations driving evolution.

On the one hand, in animal-pollinated plants, flowers are plant's reproductive organs which may interact mainly with pollinators (Fenster et al. 2004, Schiestl and Johnson 2013). Floral attributes such as flower color or flower size, may influence detection by pollinators or indicate the accessibility, quantity, or quality of resources (Spaethe et al. 2001, Schiestl and Johnson 2013). Different species of pollinators may show different preferences on floral traits, thus the variation in the pollinator community among populations may explain floral size and color divergence within plant species (Rauscher 2008; Campbell et al. 2012). However, non pollinator agents may exert selective pressures on flower attributes simultaneously to pollinators and influence flower variation (Galen 1999).

On the other hand, vegetative variation may relate to variation of physical factors, such as rainfall or temperature (Niklas et al. 2007, Milla and Reich 2011). Plant height, leaf size or the number of leaves, usually differ among populations of the same species, related to selection by environmental factors (Geber and Griffen 2003, Medrano et al. 2006, Alcántara et al. 2010). In addition, plants develop strategies to avoid herbivory or to mitigate their impact which may affect leaf and plant size and cause among population variation in non-floral traits (Díaz et al. 2001). The patterns of spatial variation of both floral and vegetative traits within species, linked to knowledge on trait functions and ecology, may indicate which agents exert natural selection on the plant species.

In addition, geographic variation in plant traits may depend on the biogeographic history of the species (Ellisson et al. 2004). Distribution of alpine species might result from isolation during the post-glacial periods within the Quaternary: during warming, alpine species of the Iberian Peninsula rise in altitude and become isolated; these

cyclical climatic changes promoted genetic isolation and diversification in the Iberian mountains (Stewart et al. 2010; Alarcon et al. 2012). If phenotypic variation results from historical processes, we might expect that floral and vegetative attributes show similar levels and patterns of variation between populations.

Gentiana lutea (Gentianaceae) shows flower color variation, from orange to yellow, solely at the western fringe of its range (SW Europe). Neither the spatial variation of floral or vegetative traits, including flower color, nor the geographical distribution of this variation was previously described in this species. We investigated whether floral variation drives differences in other phenotypic traits or whether, on the contrary, vegetative traits drag variation in floral traits. We assessed also if the phenotypic variation was related to abiotic factors (temperature, radiation and rainfall). Additionally, we described the geographic structure of flower color variation and we examined how variance of flower color is partitioned among flowers within individuals, among individuals within populations, and among populations. We assessed the relative importance of vegetative versus floral traits in population differentiation by answering the following questions: 1) Do populations of *Gentiana lutea* growing in the north of Spain differ in vegetative and floral traits? 2) Which traits allow for best discrimination among populations? 3) Are vegetative and floral differences among populations related to distance among populations? 4) Do floral traits or do vegetative traits drag the variation among populations? 5) Are floral traits or vegetative traits related to temperature, radiation and precipitation variation among populations? Investigating the geographical structure of phenotypic variation, and comparing the patterns of variation between floral and vegetative traits, provides essential information on the evolutionary process of this plant species.

3.3 MATERIALS AND METHODS

3.3.1 Field procedures

The study area covered the distribution of *Gentiana lutea* at the western half of the Cantabrian Mountains, Spain. We studied eight populations in 2010 and 12 in 2011, four of them were new. Populations were haphazardly selected along a 230 km longitudinal transect from San Mamede population in the western limit (42°12' N, 7° 30' W) until San Glorio in the eastern limit (43°04' N, 4° 45' W; Fig. 2.2 in chapter 2, page 29). All populations grow at altitudes from 1100 m to 1700 m a.s.l., on pastures.

In June, when the stem was developed but before blossom started, we chose haphazardly between 20 and 50 plants in each population. Geographical coordinates were obtained for each population with a GPS (Garmin eTrex Vista).

In July, during blossom, we measured petal length (mm), petal width (mm), stalk height (cm), leaf length (the length of the longest basal leaf, mm) and flower color for each plant individual. We counted as well the number of flowers, the number of petals and the number of basal leaves. Some plants had all their basal leaves dry so we could not measure the leaf length; as a result, the number of plants with data on floral traits is 438 and with data on vegetative traits is 429. We chose randomly three flowers in each plant to count the number of petals and to quantify the length and width of one petal per flower. We averaged the values for each plant. We considered petal length from the base of the flower until the tip of the petal and petal width on the mid part of the petal (the widest). For both measures we used a caliper to the nearest 0.1 mm. We measured the height of the stalk from the base of the plant to the top, and the length of the longest basal leaf from the insertion to the tip, with a measuring tape to the nearest 1 mm.

Flower color was recorded on ten randomly chosen flowers per plant in 2010 and on three flowers per plant in 2011 (2711 flowers from 438 plant individuals); in 2011 we reduced the number of replicates to 3 because the coefficient of variation of color variates was asymptotic after 3 flowers. Technical details on color measurement are provided in section 2.3, chapter 2, pages 29-31.

We considered as floral traits: flower color, petal length, petal width, the number of petals per flower, and number of flowers. We classified as vegetative traits: the number of basal leaves, leaf length and stalk height.

We obtained data on mean summer temperature ($^{\circ}\text{C}$), mean summer radiation ($10 \text{ kJ} / (\text{m}^2 * \text{day} * \mu\text{m})$) and mean summer precipitation (mm) for each population from the digital climatic atlas of the Peninsula Iberica (Ninyerola et al. 2005). Mean summer data come from the mean of the months of June, July and August, of between 15 and 50 years, within the period of 1951-1999 (Ninyerola et al. 2005).

3.3.2 Statistical analysis

We reduced the three color variables (L , a , and b), by principal component analysis. The first principal component (PC1) represented 63% of variance of a , b and L (eigenvalue = 1.89; $n = 2711$ flowers); thus, PC1 was the flower color variable in further analysis. Correlations between the original color variables with PC1 were: L (brightness) = 0.686, a (red related component) = -0.360, and b (yellow related component) = 0.631; meaning that low scores of the PC1 indicates orange color and high scores indicate yellow color.

We first assessed the overall phenotypic variation among populations from a multivariate perspective by means of multivariate analysis of variance (MANOVA). We analyzed separately the set of vegetative traits and the set of floral traits as dependent variables; the factors were population and year (populations were chosen following a longitudinal order thus we considered both year and population as fixed factors).

To discern which particular traits allow for differentiation among populations, we performed a discriminant function analysis (DFA), separately for each year. Note that to consider that one trait allow for discrimination among populations, two conditions are required: the variable differs among populations and the information provided is not redundant with the information provided by the other variables (Quinn and Keough 2002). We calculated the canonical variates—linear combinations of the original variables which maximize variation among populations—to explore which phenotypic traits account for most of variation among populations. The traits with the highest correlation with the canonical variates (that explain most of variation) will be the most different among populations.

We assessed whether vegetative or floral differences between populations, are correlated to distance, temperature, radiation and rainfall differences among populations, by the Mantel test (Manteltester 1.0; Bonnet and Van de Peer 2002). We calculated the matrix for the geographic distance between each pair of populations, based on the euclidean differences of their UTM coordinates and the matrix for the temperature, radiation and precipitation differences, separately, based on the absolute differences among populations. We obtained the matrix for the phenotypic differences on the set of vegetative and floral traits separately, by the Mahalanobis distances between each pair of populations. Mahalanobis distances are similar to the squared euclidean distances for multivariate populations, but, unlike the euclidean, the Mahalanobis distance takes into account the intercorrelations between the variables in the model. We run simple correlations and partial correlations—controlling for the

effect of one factor at a time. We determined the significance of Mantel test with the probability distribution based on 100000 permutations. We used data of 2011 because the number of populations was higher than in 2010.

We described the geographic structure of color variation. First, we assessed color variation among populations, by means of a general linear mixed model (GLMM): flower color (PC1) was the dependent variable, year a fixed factor and population and the population by year interaction as random factors. The dependent variable was transformed into a logarithmic function to improve the model following the AICc criterion (Quinn and Keough 2002). Additionally flower color variation was partitioned into the sources of variation related to the plant organization levels: among flowers within plant, among plants within population and among populations. The response variable was PC1 and independent variables were population and plant as random factors. For the partition of variance, we used data of populations sampled in 2010 because we measured ten flowers per plant which allowed us to better account for within plant variation.

We performed all statistical analysis, except the Mantel test (Manteltester 1.0; Bonnet and Van de Peer 2002), with Statistica for Windows, release 8 (StatSoft Inc. 2007).

3.4 RESULTS

Both floral and vegetative traits differ among populations (Table 3.1). In 2010, neither leaf length nor number of petals per flower, differed among populations but the remaining traits (flower color, petal width, petal length, number of flowers, stalk height and the number of basal leaves) allowed for discrimination between populations (Table 3.2). In 2011, populations differed in all traits analyzed. The first canonical variate (linear combination of variables that maximizes differences among populations) accounted for nearly 70% of phenotypic variation among populations in both years. Flower color and petal width were the variables that correlated the most with this first canonical variate, thus flower color and petal width are the phenotypic attributes which allow for the best discrimination among populations of *G. lutea* (Table 3.2).

Table 3.1. Multivariate phenotypic variation among populations of *Gentiana lutea*. We analyzed separately two groups of traits, floral and vegetative. N = 429 for vegetative and N = 438 for floral traits. 8 populations in 2010; 12 populations in 2011.

Type of traits	Factor	Wilks λ	df	P
Floral	Population	0.146	55;2123	0.000
	Year	0.544	5;458	0.000
Vegetative	Population	0.447	33;1247	0.000
	Year	0.995	3;423	0.565

Table 3.2. Phenotypic discrimination among populations. The variables that allow for discrimination among populations are the ones with $P < 0.05$ (in bold). We calculated the first two canonical variates (linear combinations of the original variables that maximize variation among populations) for each year, the values are the correlations between each trait and the canonical variates (canvar); underlined are correlations ≤ -0.5 and ≥ 0.5 . Number of populations in 2010 were 8; in 2011 were 12.

		2010						2011				
		Wilks λ	df	P-value	Canvar1	Canvar2	Wilks λ	df	P-value	CanVar1	CanVar2	
Floral	Flower color	0.125	7;123	0.000	-0.759	-0.163	0.1250	11;272	0.000	0.816	-0.237	
	Petal length	0.098	7;123	0.000	-0.273	-0.273	0.061	11;272	0.000	0.246	-0.567	
	Petal width	0.113	7;123	0.000	0.715	-0.161	0.075	11;272	0.000	0.619	0.076	
	N° of petals per flower	0.076	7;123	0.545	0.142	-0.017	0.051	11;272	0.014	-0.139	-0.289	
	N° of flowers	0.089	7;123	0.000	-0.316	0.165	0.051	11;272	0.014	0.118	0.287	
Vegetative	N° of basal leaves	0.097	7;123	0.000	0.334	-0.598	0.069	11;272	0.000	-0.263	-0.674	
	Leaf length	0.075	7;123	0.706	0.039	0.033	0.051	11;272	0.026	0.191	-0.039	
	Stalk height	0.093	7;123	0.000	0.358	0.718	0.064	11;272	0.000	-0.086	-0.224	
Eigen value					3.501	0.643				4.068	0.680	
Var explained ¹					0.722	0.854				0.696	0.813	

¹ The cumulative proportion of variance explained.

Distance among populations was correlated to phenotypic differences among populations: the correlation between floral differences and distance ($r = 0.778$, $P < 0.0001$) was stronger than the correlation between vegetative differences and distance among populations ($r = 0.271$, $P < 0.05$; Fig. 3.1). Results of partial correlations differed from results from simple correlations: the correlation between vegetative differences among populations and the spatial distance disappeared ($r = 0.178$, $P > 0.05$) when controlling for floral differences; the partial correlation of floral differences among populations and spatial distances remained strong after controlling for variation in vegetative traits ($r = 0.767$, $P < 0.0001$). Differences among population in abiotic factors (summer means of temperature, radiation and precipitation) were not correlated to either floral or vegetative variation among populations (results not shown).

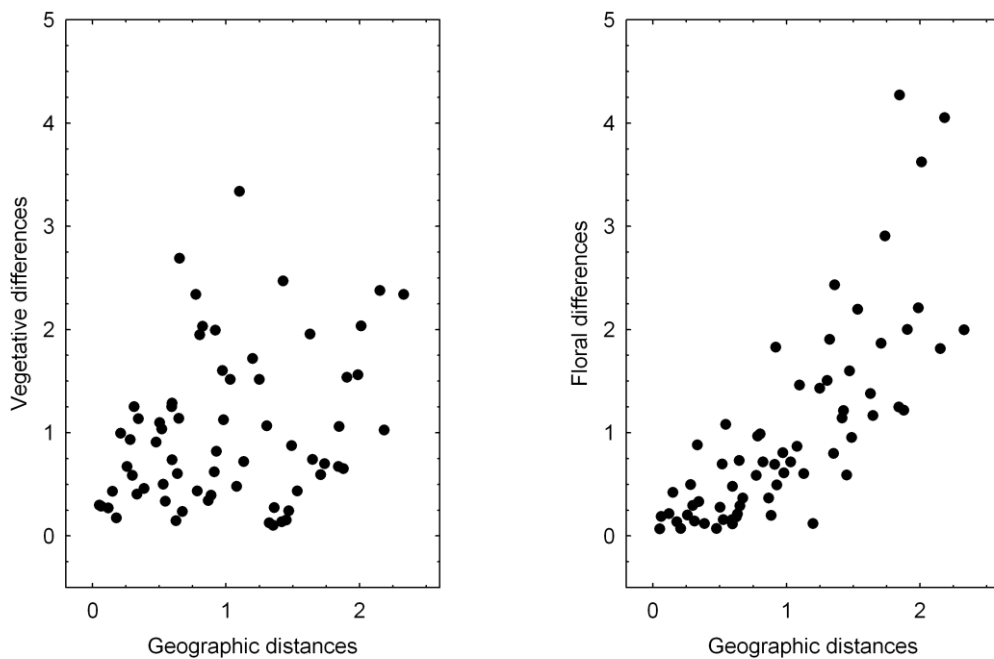


Figure 3.1. Correlation between the geographical distance among populations and the phenotypic differences on the set of floral and vegetative characters. Geographic distances were based on the euclidean differences of UTM coordinates; phenotypic differences on the set of vegetative and floral traits were calculated from the Mahalanobis distances (see methods). Note that geographic, vegetative and floral distance were relativized (divided by the average) to provide comparable values.

Flower color varied among populations (results from the GLMM; $N = 504$; $F_{11;483} = 11.54$; $P = 0.001$) and over the two study years depending on populations (population * year: $F_{8;483} = 5.57$; $P = 0.000$); but flower color means were similar both years ($F_{1;483} = 2.81$; $P = 0.132$; Fig. 3.2). Flower color among populations varied from orange to yellow towards the east of the Cantabrian Mountains (Fig. 3.2). The partition of variance showed that variation was distributed as follows: among flowers, within plants 30%; among plants, within populations 25%, and among populations 45%.

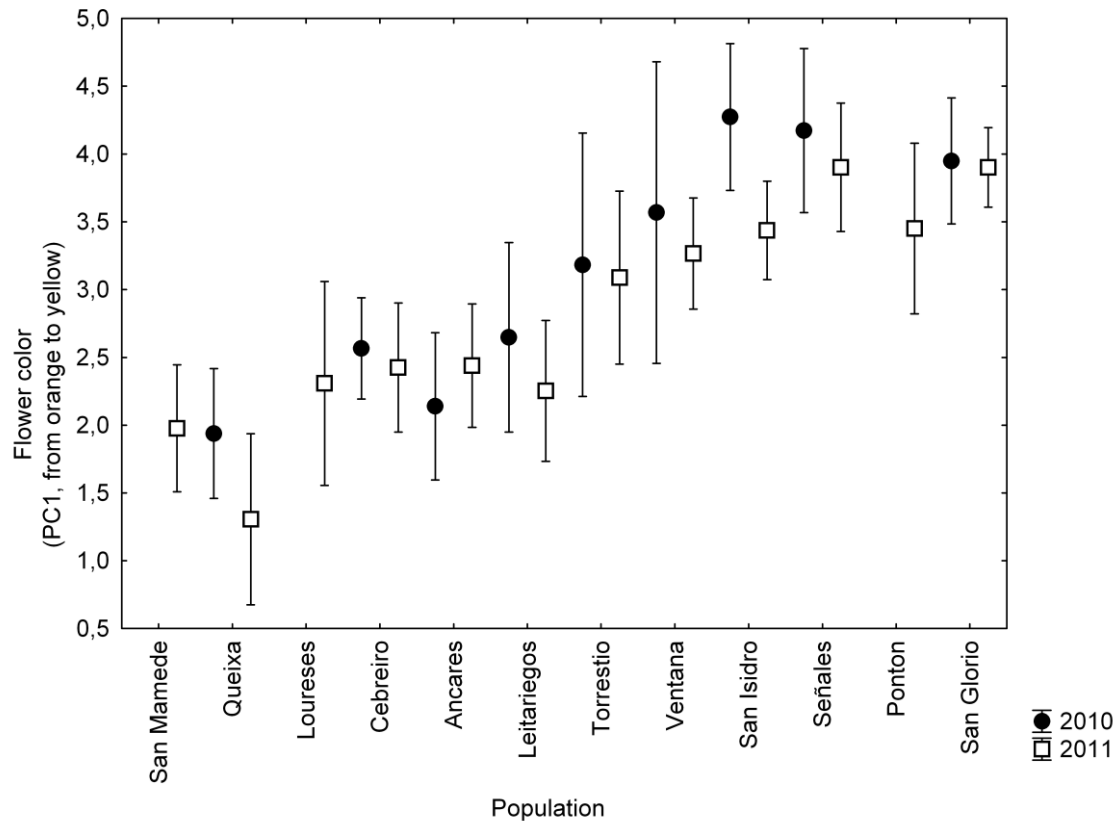


Figure 3.2. Spatial and temporal variation of flower color (PC1) in *Gentiana lutea*. Results from the generalized linear mixed model (N = 504 plants). Populations are ordered from west to east. We represented mean values and standard deviations of the observed data.

3.5 DISCUSSION

We found spatial variation of phenotypic traits among *Gentiana lutea* populations along the westernmost end of the species distribution. Populations differed in both floral and vegetative traits and these differences were not related to abiotic factors. Flower color and petal width accounted for most of the variation among populations –flower color varied from orange to yellow, according to a longitudinal pattern from west to east. Close populations presented more similar phenotypes than populations growing apart from each other. In particular, correlation between floral variation and distance between populations was more than twice the correlation of vegetative variation with distance. Even more, when controlling for floral variation, vegetative traits were not related to geographical distance. These results indicate that floral variation among the *G. lutea* populations investigated drives the phenotypic variation, mainly by variation in flower color.

If historical events were to explain phenotypic variation among populations of *Gentiana lutea* we would expect similar variation in vegetative and floral traits among populations. We found that variation in floral traits was higher than in vegetative traits, and even more, the correlation of the variation in vegetative traits among populations with distance among populations, disappeared when controlling for floral variation (most of vegetative traits were correlated to floral traits; see Table A1 in the appendix). Thus, floral and vegetative traits in this species show different patterns of variation, which seem to reflect different causes shaping them. It seems unlikely that this variation responds to random processes such as genetic drift or founder effects. Thus, phenotypic variation may occur due to natural selection.

Abiotic factors may influence the phenotypic variation on both, floral and vegetative traits (Lambrecht and Dawson 2007). Summer drought is more severe at the west of the Cantabrian Mountains (Rivas-Martínez et al. 1984), so we presumed that summer temperature, radiation or precipitation may relate to phenotypic variation among populations of *G. lutea*. However, we found that none of those factors were correlated to phenotypic variation. Thus if selective forces shape the geographic structure of variation, we presume that they might be different from abiotic forces.

Pollinators (or other selective animals interacting with flowers) might be driving the differentiation process among the *G. lutea* populations in the studied range. Flower color varied among populations (which may indicate local adaptation) and among plants within populations (which is required for natural selection to occur). Flower color is a trait genetically based (Zhu et al. 2002, 2003) and populations are genetically different (González-López et al. 2014); thus selective forces exerted by pollinators or other interacting animals may affect the geographical structure of phenotypic variation. The pollinator community varies across the Cantabrian Mountains (the study area; Obeso 1992, Ploquin et al. 2013; see Table A2 in chapter 5, pg 87); and differences in the pollinator assemblage among populations may conduct floral divergence. Covariation among floral traits might be due to the effect of correlated selection exerted by pollinators since pollinators may select on sets of floral traits (Fenster et al. 2004, Campbell 2009): combinations between flower color and floral size may influence detection of plants by pollinators and their foraging behavior (Spaethe et al. 2001).

Non-pollinator agents may exert selection also on floral traits (Strauss and Whittall 2006). During field work, we observed damage by predispersal seed predators, which may be important selective agents on floral traits (Kolb et al. 2007). Predispersal seed predators select plants during flowering; they may use the same cues as pollinators because they depend on a successful pollination to feed their larvae with the seeds. Pollinators are mutualist and seed predators are antagonist interactors. Antagonistic animals may weaken or strengthen the effect of mutualistic animals, depending on their preferences (Frey 2004, Caruso et al. 2010) and their joint effect may influence floral variation within and among populations.

In this chapter, we provide the first description of the geographic structure of the phenotypic variation in *Gentiana lutea*, focusing on flower color. We found that variation in floral traits drags variation on vegetative traits, and that abiotic factors are not correlated to phenotypic differences among populations; thus we presume that selective forces, likely biotic forces, acting on floral traits are responsible for populations' divergence. In the following chapters, we will examine the natural selection on flower color and the possible role of biotic forces (pollinators and seed predators) as selective agents.

3.6 REFERENCES

- Alarcón M, Vargas P, Sáez L, Molero J, Aldasoro J. J. 2012. Genetic diversity of mountain plants: Two migration episodes of Mediterranean *Erodium* (Geraniaceae). *Molecular Phylogeny and Evolution* 63: 866-876.
- Alcántara, J., J. Bastida, and P. Rey. 2010. Linking divergent selection on vegetative traits to environmental variation and phenotypic diversification in the Iberian columbines (*Aquilegia*). *Journal of Evolutionary Biology* 23:1218-1233.
- Armbruster, W. S., V. S. Di Stilio, J. D. Tuxill, T. C. Flores, and J. L. Velasquez Runk. 1999. Covariance and decoupling of floral and vegetative traits in nine Neotropical plants: a re-evaluation of Berg's correlation-pleiades concept. *American Journal of Botany* 86:39-55.

- Bonnet, E., and Y. Van de Peer. 2002. *zt*: a software tool for simple and partial Mantel tests. *Journal of Statistical Software* 7:1-12.
- Campbell, D. R. 2009. Using phenotypic manipulations to study multivariate selection of floral trait associations. *Annals of Botany* 103:1557-1566.
- Campbell, D., M. Bischoff, J. Lord, and A. Robertson. 2012. Where have all the blue flowers gone: pollinator responses and selection on flower colour in New Zealand *Wahlenbergia albomarginata*. *Journal of Evolutionary Biology* 25:352-364.
- Caruso, C. M., S. L. Scott, J. C. Wray, and C. A. Walsh. 2010. Pollinators, herbivores, and the maintenance of flower color variation: a case study with *Lobelia siphilitica*. *International Journal of Plant Science* 171:1020-1028.
- Díaz, S., I. Noy-Meir, and M. Cabido. 2001. Can grazing response of herbaceous plants be predicted from simple vegetative traits? *Journal of Applied Ecology* 38:497-508.
- Ellison A. M., H. L. O. Buckley, T. E. Miller, and N. J. Gotelli. 2004. Morphological variation in *Sarracenia purpurea* (Sarraceniaceae): geographic, environmental, and taxonomic correlates. *American Journal of Botany* 91: 1930-1935.
- Fenster, C. B., W. S. Armbruster, P. Wilson, M. R. Dudash, and D. James. 2004. Pollination syndromes and floral specialization. *Annual Review of Ecology, Evolution and Systematics* 35:375-403.
- Frey, F. M. 2004. Opposing natural selection from herbivores and pathogens may maintain floral-color variation in *Claytonia virginica* (Portulacaceae). *Evolution* 58:2426-2437.
- Galen, C. 1999. Why do flowers vary? *BioScience* 49:631-640.
- Geber, M. A., and L. R. Griffen. 2003. Inheritance and natural selection on functional traits. *International Journal of Plant Sciences* 164:S21-S42.
- González-López, O., C. Polanco, Z. György, A. Pedryc, and P. A. Casquero. 2014. Genetic variation of the endangered *Gentiana lutea* L. var. *aurantiaca* (Gentianaceae) in populations from the northwest Iberian Peninsula. *International Journal of Molecular Sciences* 15:10052-10066.
- Gould, S. J., and R. F. Johnston. 1972. Geographic variation. *Annual Review of Ecology and Systematics* 3:457-498.
- Kolb, A., J. Ehrlén, and O. Eriksson. 2007. Ecological and evolutionary consequences of spatial and temporal variation in pre-dispersal seed predation. *Perspectives in Plant Ecology, Evolution and Systematics* 9:79-100.
- Lambrecht, S. C., and T. E. Dawson. 2007. Correlated variation of floral and leaf traits along a moisture availability gradient. *Oecologia* 151:574-583.
- Linhart, Y. B., and M. C. Grant. 1996. Evolutionary significance of local genetic differentiation in plants. *Annual Review of Ecology and Systematics* 27:237-277.
- Medrano, M., M. C. Castellanos, and C. M. Herrera. 2006. Comparative floral and vegetative differentiation between two European *Aquilegia* taxa along a narrow contact zone. *Plant Systematics and Evolution* 262:209-224.
- Milla, R., and P. B. Reich. 2011. Multi-trait interactions, not phylogeny, fine-tune leaf size reduction with increasing altitude. *Annals of Botany* 107:455-465.
- Niklas, K. J., E. D. Cobb, U. Niinemets, P. B. Reich, A. Sellin, B. Shipley, and I. J. Wright. 2007. "Diminishing returns" in the scaling of functional leaf traits across and within species groups. *Proceedings of the National Academy of Sciences of the United States of America* 104:8891-8896.

- Ninyerola, M., J. M. Roure, and X. P. Fernández. 2005. Atlas climático digital de la Península Ibérica: metodología y aplicaciones en bioclimatología y geobotánica. Universidad Autónoma de Barcelona, Bellaterra, Barcelona, Spain.
- Obeso, J. R. 1992. Geographic distribution and community structure of bumblebees in the northern Iberian Peninsula. *Oecologia* 89:244-252.
- Ploquin, E. F., J. M. Herrera, and J. R. Obeso. 2013. Bumblebee community homogenization after uphill shifts in montane areas of northern Spain. *Oecologia* 173:1649-1660.
- Quinn, G. P., and M. J. Keough. 2002. Experimental Design and Data Analysis for Biologists. Cambridge University Press, Cambridge, United Kingdom.
- Rausher, M. D. 2008. Evolutionary transitions in floral color. *International Journal of Plant Sciences* 169:7-21.
- Rivas-Martínez, S., T. E. Díaz, J. A. F. Prieto, J. Loidi and A. Penas. 1984. La Vegetación de la Alta Montaña Cantábrica: Los Picos de Europa. Ediciones Leonesas. León.
- Schiestl, F. P., and S. D. Johnson. 2013. Pollinator-mediated evolution of floral signals. *Trends in Ecology and Evolution* 28:307-315.
- Schluter, D. 2009. Evidence for ecological speciation and its alternative. *Science (New York, N.Y.)* 323:737-741.
- Spaethe, J., J. Tautz, and L. Chittka. 2001. Visual constraints in foraging bumblebees: flower size and color affect search time and flight behavior. *Proceedings of the National Academy of Sciences of the United States of America* 98:3898-3903.
- StatSoft, I. 2001. STATISTICA (data analysis software system), version 6. Tulsa, USA.
- Stewart J. R., A. M. Lister, I. Barnes, and L. Dalén. 2010. Refugia revisited: individualistic responses of species in space and time. *Proceedings of the Royal Society of London B Biology* 277: 661-671.
- Strauss, S. Y., and J. B. Whittall. 2006. Non-pollinator agents of selection on floral traits. Pages 120-138 *In* Harder, L. D., and S. C. H. Barrett, eds. *Ecology and Evolution of Flowers*, Oxford University Press, New York.
- Zhu, C., S. Yamamura, H. Koiwa, M. Nishihara, and G. Sandmann. 2002. cDNA cloning and expression of carotenogenic genes during flower development in *Gentiana lutea*. *Plant Molecular Biology* 48:277-285.
- Zhu, C., S. Yamamura, M. Nishihara, H. Koiwa, and G. Sandmann. 2003. cDNAs for the synthesis of cyclic carotenoids in petals of *Gentiana lutea* and their regulation during flower development. *Biochimica et Biophysica Acta (BBA)-Gene Structure and Expression* 1625:305-308.

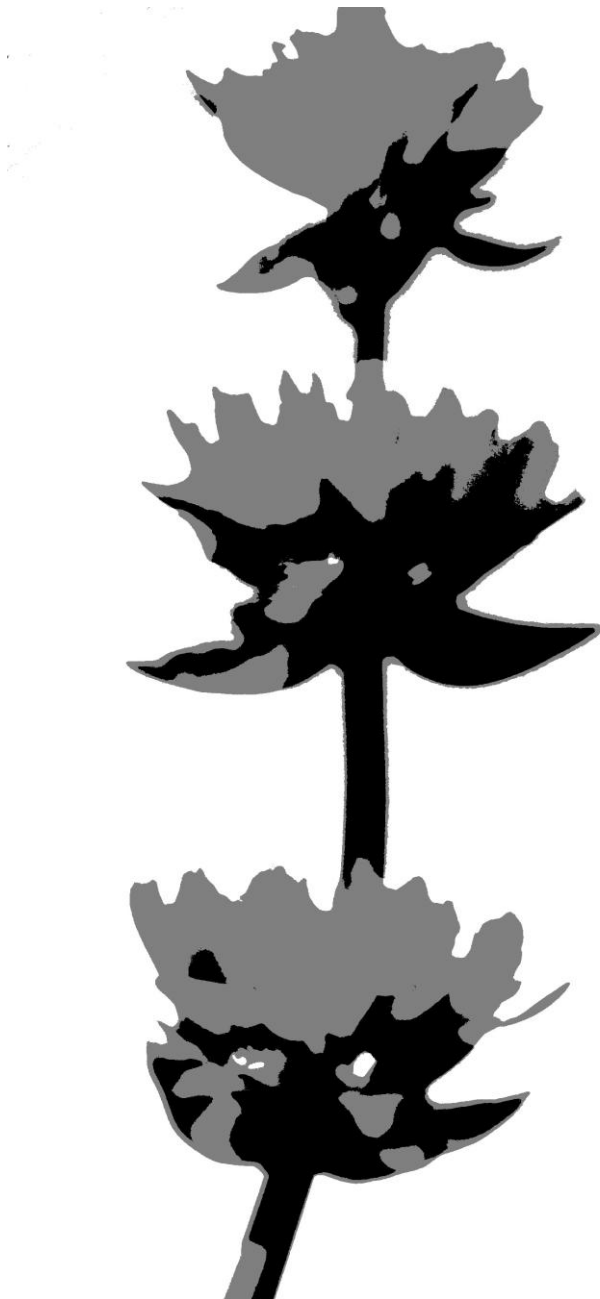
3.7 APPENDIX

Table A1. Phenotypic trait correlations. Above diagonal the correlation coefficient (r), below diagonal the p-values. In bold are correlations with p-value < 0.05. N = 429.

	Flower color	Petal length	Petal width	N° petals ¹	N° of flowers	N° of leaves ²	Leaf length	Stalk height
Flower color	—	-0.118	-0.493	-0.092	0.166	-0.193	0.005	-0.092
Petal length	0.014	—	0.524	-0.011	-0.007	0.015	0.270	0.338
Petal width	0.000	0.000	—	-0.054	-0.140	0.159	0.115	0.267
N° of petals ¹	0.058	0.820	0.268	—	0.036	0.175	0.127	0.083
N° of flowers	0.001	0.882	0.004	0.456	—	0.312	0.230	0.262
N° of leaves ²	0.000	0.757	0.001	0.000	-0.049	—	0.097	-0.105
Leaf length	0.913	0.000	0.017	0.008	0.000	0.045	—	0.410
Stalk height	0.057	0.000	0.000	0.086	0.000	0.030	0.000	—

¹ N° of petals per flower. ² N° of basal leaves.





4. ARE POLLINATORS AND SEED
PREDATORS SELECTIVE
AGENTS ON FLOWER COLOR IN
Gentiana lutea L.?



4.1 ABSTRACT

Animals which interact with plants often cause selective pressures on plant traits. Flower color variation within a species might be shaped by the action of animals feeding on the plant species. Pollinators might exert natural selection on color if flower color is related to their foraging efficiency. For example, some pollinator species might require more time to detect particular colors. If that is the case, flower color might have evolved as a pollination exploitation barrier—ensuring that flowers are more visited by the most efficient pollinators. In addition, non-pollinator agents such as predispersal seed predators may select on flower color, if color indicates food resources (seeds) or if color is related to deterrent compounds. We address selection on flower color in a population of *Gentiana lutea* where color varies among individuals from yellow to orange. We hypothesize that opposed selection from mutualists (pollinators) and antagonists (predispersal seed predators) maintains flower color variation in this population. By means of path analysis we addressed the role of both interactors in flower color selection. We found that selection acts on flower color, mediated by both pollinators and seed predators. Both agents favored yellow-flowered individuals, thus selection by pollinators and seed predators does not explain flower color variation in this population.

4.2 INTRODUCTION

Flower color evolution is traditionally linked to direct selection exerted by pollinators since it is related to quality or quantity of rewards (Fenster et al. 2004, Rausher 2008) and to plant detectability (Spaethe et al. 2001). Flower color variation may have evolved as a pollination exploitation barrier if particular colors impede or restrict the access to less effective pollinators and increase the visits to the most effective pollinators (Rodríguez-Gironés and Santamaría 2004). Some pollinator species require more time to detect particular colors, decreasing their foraging efficiency. For instance, most bees and bumblebees lack red-color receptors (Chittka and Waser 1997, Briscoe and Chittka 2001), which increase their time to detect red flowers (Jones and Reithel 2001, Spaethe et al. 2001). It is likely that pollinators increase their visits to the most detectable plants (in the hypothetical scenario in which other factors affecting pollinator foraging efficiency are similar among colors; Rodríguez-Gironés and Santamaría 2004). In turn, plants benefit from restricting their access to the most effective pollinators because it may increase the conspecific pollen transfer. As a consequence, changes in the community of pollinators may cause the evolution of species through flower color variation (Streisfeld and Kohn 2007, Rausher 2008).

Flower color variation may be also under selective pressures exerted by herbivores (Strauss and Whittall 2006). Some pigments, such as anthocyanins, share common biosynthetic pathways to defensive compounds (Lev-Yadun and Gould 2009). Thus, flower color may function as a signal of toxicity for herbivores and herbivores may avoid plants with particular flower colors (Strauss et al. 2004). Concretely, antagonist animals which oviposit into flowers—larvae are seed predators—may affect flower color variation. Seed predators reduce the seed production, affecting plant fitness (Kolb et al. 2007) and, since adults oviposit prior to the end of flowering, they might select on floral attributes. Flower color might affect their choices if it is related to defensive compounds (Irwin et al. 2003, Strauss et al. 2004) or to food resources (otherwise, seeds; Caruso et al. 2010).

Seed predators may strengthen or weaken the effect of pollinators. Antagonist seed predators and mutualist pollinators have opposite effects on plant fitness. Thus, if their preferences differ, selection by seed predators may strengthen the selection exerted by

pollinators (Fineblum and Rausher 1997, Caruso et al. 2010). For example, if flower color preferred by pollinators is related to plant defenses, seed predators may avoid the most pollinated plants: this non opposed selection may reduce the flower color variation. On the contrary, if preferences of mutualist and antagonist animals are similar, opposed selection may occur (Frey 2004, Strauss and Irwin 2004). This could be the case if the most pollinated flowers hold more seeds and thus are more appealing for seed predators. Similar preferences translate in opposed selection, which may maintain color variation.

Flower color is commonly correlated with other phenotypic traits (in fact, floral trait combinations regarding color, shape and nectar production, define pollination syndromes; Fenster et al. 2004). In case flower color correlates to other plant attributes, selection might act indirectly on flower color throughout the correlated traits (Armbruster 2002, Campbell 2009). For instance, indirect selection on flower color might happen if; pollinators select on flower size and color covaries with it (Gómez 2000, Carlson and Holsinger 2013), or if flower pigments are related to plant defenses and herbivores avoid plants with those components (Armbruster 2002, Irwin et al. 2003, Frey 2004, Strauss et al. 2004).

In this chapter, we describe natural selection on flower color in a population of *Gentiana lutea*. This species shows typically yellow corollas all over its distribution but at the western edge of its range (SW Europe; Iberian Peninsula), color changes into brick-orange. Flower color in this species is genetically based (Zhu et al. 2002, 2003). The studied population shows flower color variation, from brick-orange to pale-yellow flowered individuals. Different bumblebees species pollinate *G. lutea* (Rossi 2011) and in the studied population, seed predators belong to Lepidoptera (personal observations).

We hypothesize that opposed selective pressures exerted by pollinators and seed predators on flower color may maintain *G. lutea* color variation in this population. On the one hand, we presume that yellow-flowered plants may receive more visits when compared to the orange-flowered plants: redness might act as a pollination barrier decreasing detection of plants by pollinators. Also, we predict that yellow-flowered plants may suffer higher predation rates. Yellow-flowered plants may produce more seeds, as a consequence of higher pollination, thus, be more appealing to seed predators. Similar color preferences and opposite effects on fitness of pollinators and seed predators would cause opposed selective pressures—which may explain the variation in flower color within the population.

To test previous hypotheses we need to assess the effect of flower color on pollinator visits and seed predation rates, as well as the effects of both interactors on plant fitness. We used structural equation modeling (SEM) to analyze simultaneously the following relationships: (1) flower color – other plant traits, (2) flower color – interaction with animals (both pollinators and seed predators), (3) interaction with animals – plant fitness and (4) flower color – plant fitness. By introducing other plant traits into the models, we test for indirect selection on flower color through correlated traits. With this approach, we study natural selection on flower color within a population of *Gentiana lutea*, and the role of the animal interactors as selective agents. Specifically we tested if (1) flower color determines the success of pollination and (2) flower color determines the rates of seed predation, if (3) flower color is related to plant reproductive output (there is selection on flower color) and finally if (4) selection on flower color is mediated by the action of pollinators and seed predators on flower color or through correlated plant traits.

4.3 MATERIALS AND METHODS

4.3.1 Field procedures

We carried out the study at a *Gentiana lutea* population situated in Torrestío, León, Spain (43° 03' N; 6° 04' W; 1600 m a.s.l). Temperatures range from minimum average of 2 °C during winter to maximum average of 22 °C during summer; precipitation reaches maximum during winter with 175 mm and minimum during summer with 75 mm; mean annual rainfall equals 1100 mm (Moreno et al. 1990). The number of gentian individuals growing in Torrestío is up to 2000, spanning about 4 ha, distributed over pastures.

We studied 95 *Gentiana lutea* individuals. Plants present flowers only for several days a year, thus, to increase sample size, we sampled the plant population in two different seasons. We selected plants haphazardly both seasons, instead of repeating the same individuals, because *G. lutea* individuals do not flower every year. We conducted all field procedures from June to August, in 2011 and in 2012. We first visited the populations when the stems were developed, before blooming started; we choose haphazardly 50 plants in 2011 and 45 plants in 2012, separated one from each other at least 3 m. Of the 95 plants studied, 45% were orange-flowered plants, 35% were yellow-flowered ones and 20% presented an intermediate coloration.

For each plant, we measured several attributes. We measured flower color (see pages 29-31 in chapter 2 for technical details), petal length and petal width. Petal length and width were measured with a caliper to the nearest 0.1 mm, on each of three petals belonging to three randomly chosen flowers per plant. Additionally, we measured other plant traits such as the height of the stalk and the length of the longest basal leaf, with a measuring tape to the nearest 1 mm, and we counted the number of flowers.

We used principal component analysis to reduce the three color features (a, b and L). The first principal component represented a 72% of variance (PC1; eigenvalue = 2.17), so we considered that it was a good surrogate of color and we used it in further analysis. Factor coordinates of the original variables on PC1 were L = 0.93; a (red) = -0.68; b (yellow) = 0.91; which means that PC1 increases from orange to yellow.

We performed pollination censuses to measure the number of pollinator visits by minute for each plant. We did not observe differences in flower phenology among plants with different flower colors. Thus, we randomized the order of censuses across the day and all plants were censused each day. We counted all insects visiting flowers and making contact with the anthers and stigmas. We performed 10 censuses of 2 minutes per plant during 2011, and 15 censuses of 2 minutes per plant in 2012 (38.6 total hours of observations). We carried out several short censuses, instead of fewer and longer, to achieve an even distribution between 1000h and 1900h (Greenwich Mean Time), and thus, avoid confounding factors due differences in the activity of pollinators across daytime. Temperatures during censuses ranged between 12° C and 26° C and no censuses were performed during windy conditions or rain; all censused plants had over 80% of flowers opened.

When fruits were ripen, but not yet opened, we estimated the rates of the escape from predispersal seed predation (hereafter, escape from predation). We selected randomly from seven to ten fruits in each plant and we search for signals of damage by larvae. Previously we had checked that the signals, such as holes or rotting, were unequivocal for predation. We calculated the escape from predation as the percentage of fruits that were not affected by seed predators.

Finally, in a later visit to the population we counted the total number of fruits of each plant and we collected from 7 to 13 fruits per individual (fruits were not yet opened thus all seeds produced were within them). We counted the number of viable seeds in each fruit. It was impractical to us to count all seeds produced by each plant—plants had on average 79 fruits and each fruit a mean of 63 seeds; this would mean counting around 5000 seeds per plant. We estimated the total seed production of one plant by multiplying the number of fruits of each plant, times the average of viable seeds per fruit. We used production of viable seeds as a proxy for plant fitness. Seed production is an accepted surrogate of total fitness in long-lived organisms since measuring total fitness is often not possible (see Kingsolver et al. 2001).

4.3.2 Statistical analysis

We studied natural selection on flower color, and the role of animal herbivores and pollinators as selective agents by means of path analysis. By this means we can test simultaneously the following relationships (1) flower color – other plant traits, (2) flower color – interaction with animals (both pollinators and seed predators), (3) interaction with animals – plant fitness and (4) flower color – plant fitness. We first tested for temporal variation in these relationships by means of generalized linear models. If there is no temporal variation, we can join data of both sampling seasons to improve the predictive power of the path analysis. Seed production, pollinator visits and the rate of escape from predation were included as dependent variables (a separate model for each variable) and as explanatory variables; plant traits, season, and season * trait interactions. We did not detect any significant season*trait interaction, which indicates that the effects of flower color and other plant traits on pollinator visits, escape from predation and fitness did not change among seasons (data not shown). Based on those results and with the aim of improving the predictive power of our analysis, we joint data from both sampling seasons to perform the path analysis.

We built an a priori basic saturated path model with the following relationships: plant traits (flower color, petal length, petal width, leaf length, number of flowers and stalk height) directly connected to pollinator visits, escape from predation and fitness; and pollinator visits and escape from predation were connected to fitness. We included also the covariances between plant traits. We tested the goodness of fit of the models by means of maximum-likelihood estimation (MLE) on the variance-covariance matrix. A non-significant goodness of fit test indicates that the model is a good description of the observed covariance among the variables (Grace 2006). We used the generalized least squares shifting to maximum likelihood method to calculate the correlation parameters. Structural equation modeling was performed with SEPATH procedure in Statistica for Windows, release 8 (StatSoft Inc. 2007).

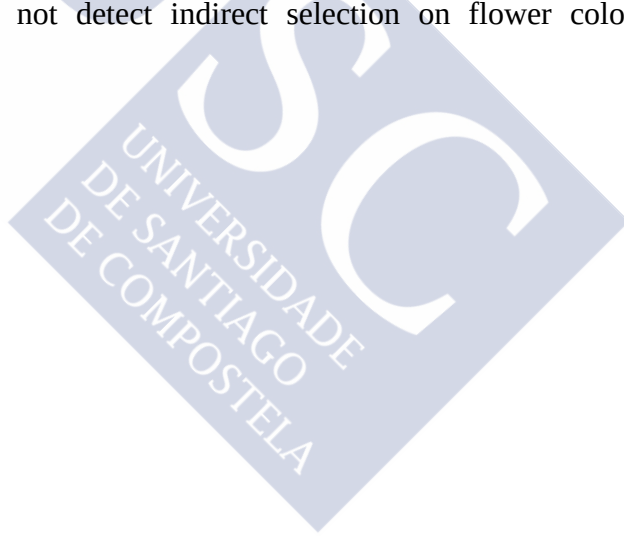
We addressed total selection and direct selection on flower color following the standard procedures described by Lande and Arnold (1983). We analyzed total selection on color (and the other traits) by simple linear regressions; relative fitness was the independent variable and each trait (standardized) the predictive variable. The total selection differentials (S) are the standardized coefficient of the regression. To assess the direct selection on color, we analyzed the effect of flower color and the remaining phenotypic traits (leaf length, stalk height, petal width, petal length and flower number; all standardized) on the relative fitness; by means of a multiple regression model. The direct selection gradients (β) are the standardized partial regression coefficients.

4.3.3 Results

We recorded a total of 1108 pollinator visits during 38.6 hours of censuses. The main visitors were bumblebees (95%), although honeybees (3%) and other

Hymenoptera (2%) also visited plants. Among bumblebees, we identified 7 species: *B. terrestris* (52% of visits); *B. pratorum* (33%), *B. lucorum* (3%), *B. mesomelas* (3%), *B. hortorum* (1%), *B. jonellus* (1%), and *B. wurflenii* (1%). Seed predators belong to Lepidoptera larvae (M. González, personal communication); we lack more specific information on the identity of seed predators because during field work we did not observe any insect while ovipositing. Plants had on average ($\pm$ standard deviation) 79 ($\pm$ 30) fruits. We counted seed production from a total of 835 fruits, which had an average of 63 ($\pm$ 24) seeds each. Some plants were trampled, so the number of plants studied decreased in successive visits to the field: 94 plants have data on pollinator visits and the escape from predation; 76 plants have data on seed production.

The basic saturated path model explained successfully the covariation among the variables (ML Chi-Square: 1.374; d.f. = 1; $P = 0.241$). Flower color affected both pollinator visits and the escape from seed predators. Both agents favored yellowness: pollinators visited more the yellow-flowered plants and seed predators avoided plants with yellow coloration (Fig. 4.1a, 4.1b; Fig. 4.2). Both pollinators and seed predators (predators marginally; $P = 0.052$; $r = 0.161$) affected the production of viable seeds, with positive and negative effects respectively (Fig. 4.2). Interestingly, flower color and seed production did not present a direct link, which means that selection on flower color, in this population, is totally driven by the effect of pollinators and seed predators (Fig. 4.1c; Fig. 4.2). Flower color is only correlated with leaf length; plants with longer leaves showed increased redness. Leaf length did not affect pollinators, seed predators or fitness; thus, we did not detect indirect selection on flower color within this population.



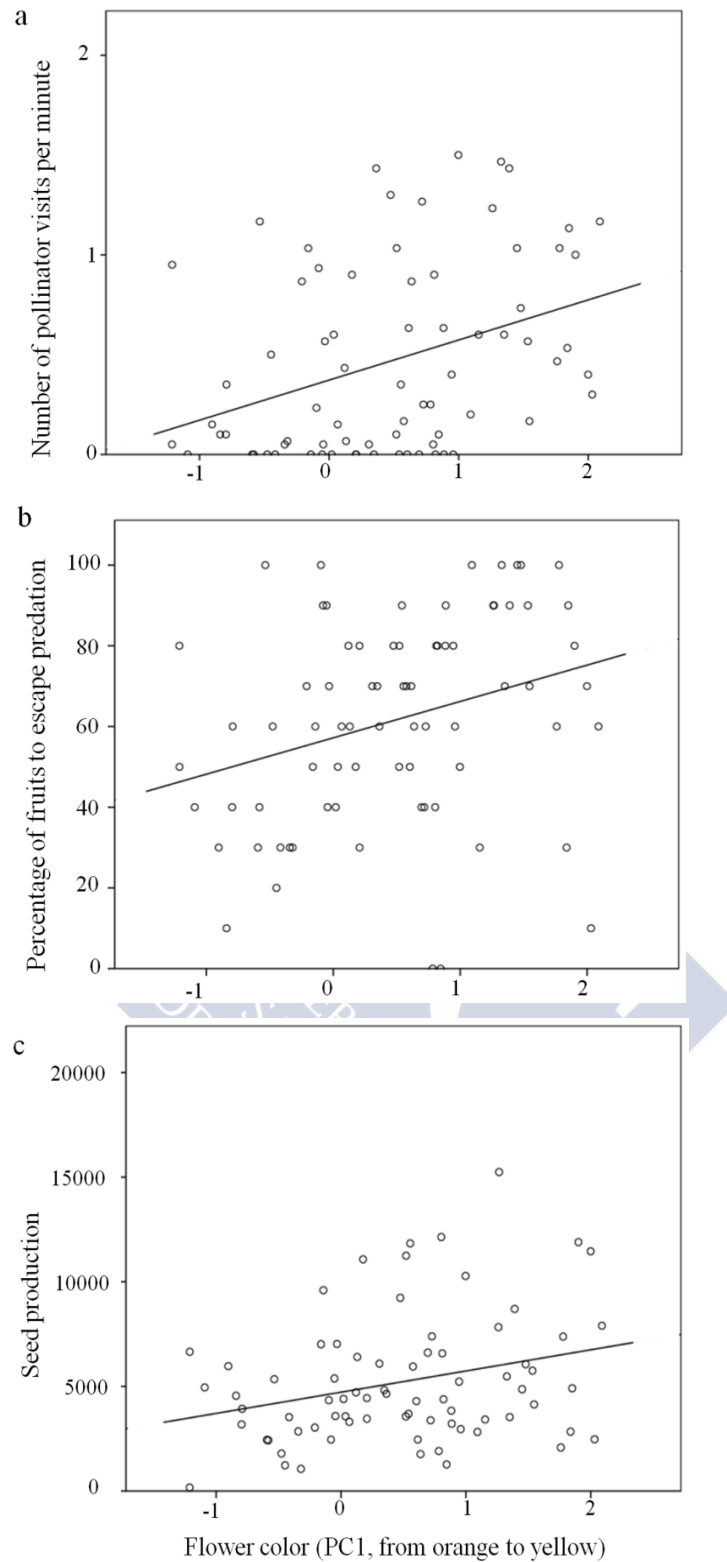


Figure 4.1. Observed values of flower color (PC1) and (a) pollinator visits, (b) escape from predation, and (c) seed production.

Besides color, most of the phenotypic traits were positively correlated among them. Flower size (petal width and petal length) is positively correlated with both; plant size (stalk height and leaf length) and number of flowers. Otherwise, larger plants had more and bigger flowers (Table 4.1).

Table 4.1. Phenotypic correlations between plant traits. N = 76. Above diagonal we show the coefficient of Spearman correlations, below diagonal are P values. In bold are correlations with P values < 0.05.

Factor	Flower color	Leaf length	Petal length	Petal width	Stalk height	Flower number
Flower color	--	-0.240	-0.184	-0.222	-0.103	0.085
Leaf length	0.037	--	0.287	0.368	0.524	0.268
Petal length	0.111	0.012	--	0.373	0.334	0.038
Petal width	0.054	0.001	0.001	--	0.287	0.001
Stalk height	0.374	0.000	0.003	0.012	--	0.258
Flower number	0.463	0.019	0.742	0.991	0.024	--

The number of flowers and the height of the stalk affected plant fitness both directly and through the effect of pollinators and seed predators. Plants with more flowers were more visited by pollinators and they were less attacked. As a result, they produced larger amounts of seeds. Plants holding more flowers tended to be higher, and coherently with previous results, higher plants produced more seeds. But, unexpectedly, higher plants received less pollinator visits and were more affected by seed predators (Fig. 4.2). This may be explained by indirect selection on the height of the stalk: since taller plants had more flowers, the strong positive effect of the number of flowers on seed production counteracted the negative effect of stalk height on pollination and seed predation escape. As a result of this indirect selection, stalk height has a total positive effect on seed production (Fig. 4.2; Table 4.2).

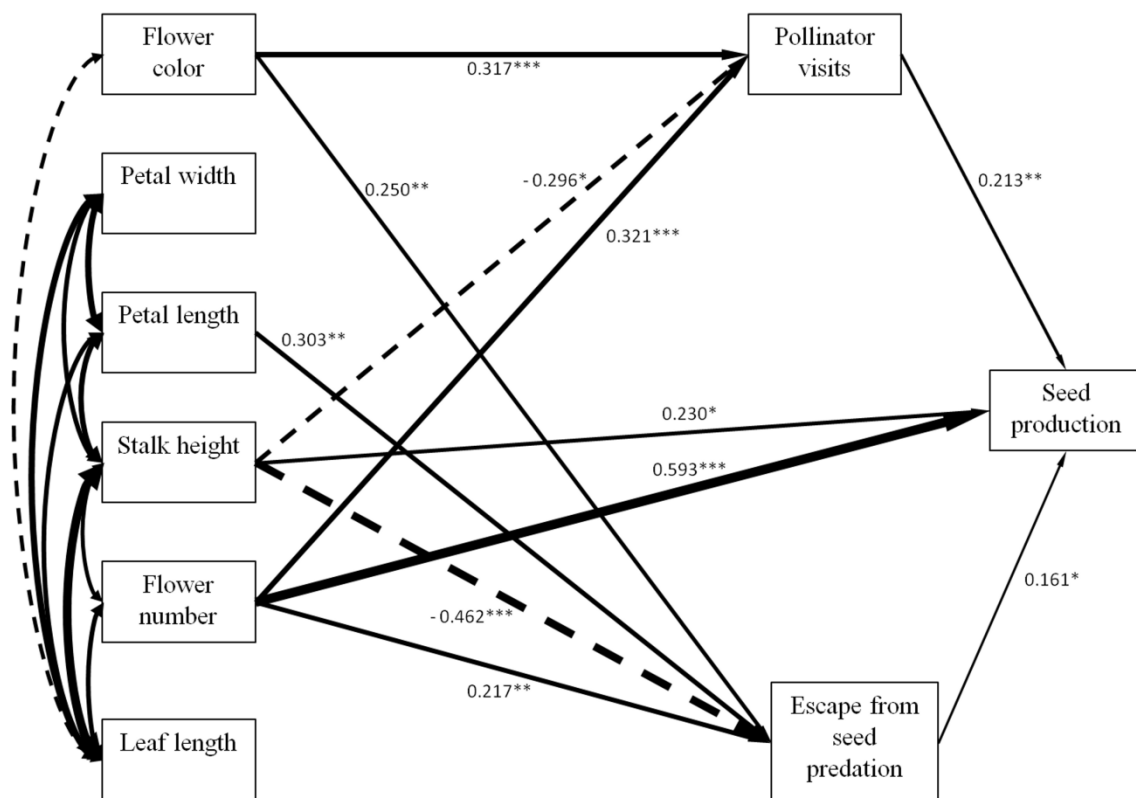


Figure 4.2. Representation of the path diagram with the significant relations among phenotypic traits, animal interactors and fitness. We show the correlation coefficients for each significant path, but for correlations among (all correlations are given in Table 4.1). The widths of the lines represent the relative strength of the path. Solid lines indicate positive effects, broken lines indicate negative effects, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

We detected significant total selection differentials and direct selection gradients on flower color: yellow-flowered plants are positively selected. The coefficient of total selection (S) was similar to the coefficient of direct selection (β), indicating that indirect selection on flower color through correlated traits is small (Table 4.2).

Table 4.2. Standardized total selection differential (S) and direct selection gradients (β). N = 75.

Factor	S (s. d.)	F	P	β (s. d.)	F	P
Flower color	0.281 (0.112)	6.235	0.015	0.238 (0.081)	8.652	0.004
Leaf length	0.147 (0.117)	1.547	0.217	-0.180 (0.097)	3.462	0.067
Petal length	0.273 (0.113)	5.875	0.017	0.207 (0.034)	5.688	0.012
Petal width	0.157 (0.115)	1.846	0.178	0.156 (0.087)	3.225	0.077
Stalk height	0.357 (0.109)	10.618	0.002	0.134 (0.095)	2.025	0.159
Flower number	0.682 (0.086)	63.330	0.000	0.651 (0.083)	60.97	0.000

4.4 DISCUSSION

We found selection on flower color mediated by pollinators and seed predators. The values of phenotypic selection found in this study are high (almost double, $\beta = 0.24$ and $S = 0.28$) compared to the absolute average values of selection reported by the review of Kingsolver et al. (2001). Pollinators preferred yellow-flowered plants while seed predators avoided them; thus, yellow-flowered plants were positively selected. We expected that seed predators would exert opposed selection to pollinators if predators oviposit on flowers preferred by pollinators (to ensure food resources for their larvae because flowers with higher pollination rates are expected to contain more seeds). Contrary to our expectations, seed predators preferred to oviposit in orange flowering plants. Thus, seed predators reinforced the effect of pollinators and the yellow-flowered plants resulted positively selected by both agents. Therefore, flower color variation in this population is not explained by current selection exerted by pollinators and seed predators.

Since yellow-flowered plants are positively selected in the population of study, we might expect a higher frequency of the yellow coloration. However, the yellow-flowered plants do not outnumber the orange-flowered plants (45% plants have orange flowers, 35% have yellow ones and 20% presented an intermediate coloration). A number of reasons might explain flower color variation in this population—despite the positive selection of yellow flowering plants by pollinators and seed predators.

The community of pollinators changed over the past decades at the Cantabrian Range (Ploquin et al. 2013). Temporal changes in the community composition may change the direction and magnitude of selective pressures (Eckhart 1992, Frey 2004, Price et al. 2005), contributing to flower color variation. Redness may difficult detectability by most of bumblebee species (Briscoe and Chittka 2001). Among the pollinator species visiting *G. lutea* plants, the most abundant pollinators (*Bombus terrestris* and *B. pratorum*) manifest low sensibility for detecting red colors (Peitsch et al. 1992, Briscoe and Chittka 2001), which may explain that increasing redness reduces pollination success. But, we presume that there might be pollinator species with higher sensibility on red colors that may increase fitness of the orange-flowered *G. lutea* plants. In fact, *Bombus lapidarius* is present in other *G. lutea* populations (personal observations, which are entirely of orange coloration) and this species shows sensitivity for red signals (although it lacks a red color receptor; Kugler 1943, Peitsch et al. 1992).

Those pollinator species with a higher sensibility or innate preferences for red colors, may be present some years; during those years, it might happen that orange-flowered plants increase their fitness. Additionally, gene flow from nearby populations where those red-sensitive pollinator species prevail, could contribute to the maintenance of orange coloration. However, we expect gene flow to be very low since the population is located in a valley surrounded by mountains, isolated from other populations.

Variation in flower color in this population might also be due to selective pressures exerted by other herbivores. Selection on flower color mediated by herbivores was found for flowers with anthocyanin pigments –which provide colorations from red to blue (for instance on *Raphanus sativus*, see Irwin et al. 2003; on *Claytonia virginica*, Frey 2004; on *Lobelia siphilitica*, Caruso et al 2010; or on *Protea aurea*, Carlson and Holsinger 2013); those pigments are related to secondary deterrent compounds (Lev-Yadun and Gould 2009). Yellow-red variation in floral coloration is caused by carotenoids in *G. lutea* (Zhu et al. 2002) and other species (Tanaka et al. 2008, Zhu et al. 2010). Although, to the best of our knowledge, the relationship of carotenoids with defensive compounds is unknown (Schaefer and Rolshausen 2006) and further research is required to understand the relationship of yellow and orange flower pigmentation with plant defensive compounds.

Other possible explanation of the flower color variation within the studied population is that selective forces may influence flower color, by exerting their influence during other periods of the life cycle, between seed production and the development of fertile progeny. For example, Streisfeld and Kohn (2007) found that the red-flowered plants of *Mimulus aurantiacus* survived better and grew larger than the yellow-flowered ones.

Environmental abiotic factors such as rainfall and temperature may affect germination and seedling survival: genes responsible for pigments may influence physiological traits related to drought and heat stress resistance (Schemske and Bierzychudek 2007, Arista et al. 2013). Particularly, some carotenoids protect plants against an excess of sunlight (Green and Durnford 1996) which may affect plants' grow under different sunlight conditions. We may explain the frequency of the orange-flowered *G. lutea* plants as a result of selective pressures exerted by environmental factors: it may happen that although yellow-flowered plants sire more seeds, orange-flowered plants survive better. In addition, color variation may relate to soil properties such as pH or aluminum concentration (Ma et al. 2001, Yoshida et al. 2003). However, we studied some edaphic (in chapter 2, see pages 36-37) and climatic factors (in chapter 3) and we have not found relationships between soil pH and plant floral color. Neither have we done between temperature, radiation and precipitation and population vegetative or floral traits. Thus, it seems unlikely that abiotic factors are driven selection on flower color within this population.

Even more, although we did not detect indirect selection on color through correlated traits, it is still possible that other traits (different to the ones measured here) may alter the relationship between color and fitness. For example, floral scent can influence bumblebee's choices and color discrimination (Kunze and Gumbert 2001); color may act as a clue indicating the quality of nectar (Rodríguez-Gironés and Santamaría 2004) and nectar rewards may affect also pollinator choices (Rodríguez-Gironés and Santamaría 2005, Veiga et al. 2013). In such cases indirect selection may occur. However, and due to the similar values of the total selection coefficient (S) and

the direct selection gradient (β) on flower color found here (Table 4.2) we expect that indirect selection on color within this population is not very important.

Flower pigmentation in this species is genetically based (Zhu et al. 2002, 2003), resulting from the regulation of genes involved in the synthesis or storage of carotenoids (Zhu et al. 2010). Those genes may affect the carotenoid content and quantity (Tanaka et al. 2008), providing yellow/orange color variation—such it happens in other species; see for example Moebs et al. (2001) and Bradshaw and Schemske (2003). However, yellow/orange variation may also be due to anthocyanin pigments: Tanaka and Ohmiya (2008) hypothesized for other species of the genera *Gentiana* that the genetically based inability to accumulate pelargonidin (an anthocyanin pigment) impeded to produce orange coloration.

We conclude that flower color is selected in the studied *G. lutea* population by both pollinator and seed predator animals. Both interactors favour yellow flowering individuals: Thus, their combined action does not explain flower color polymorphism in this population.

4.5 REFERENCES

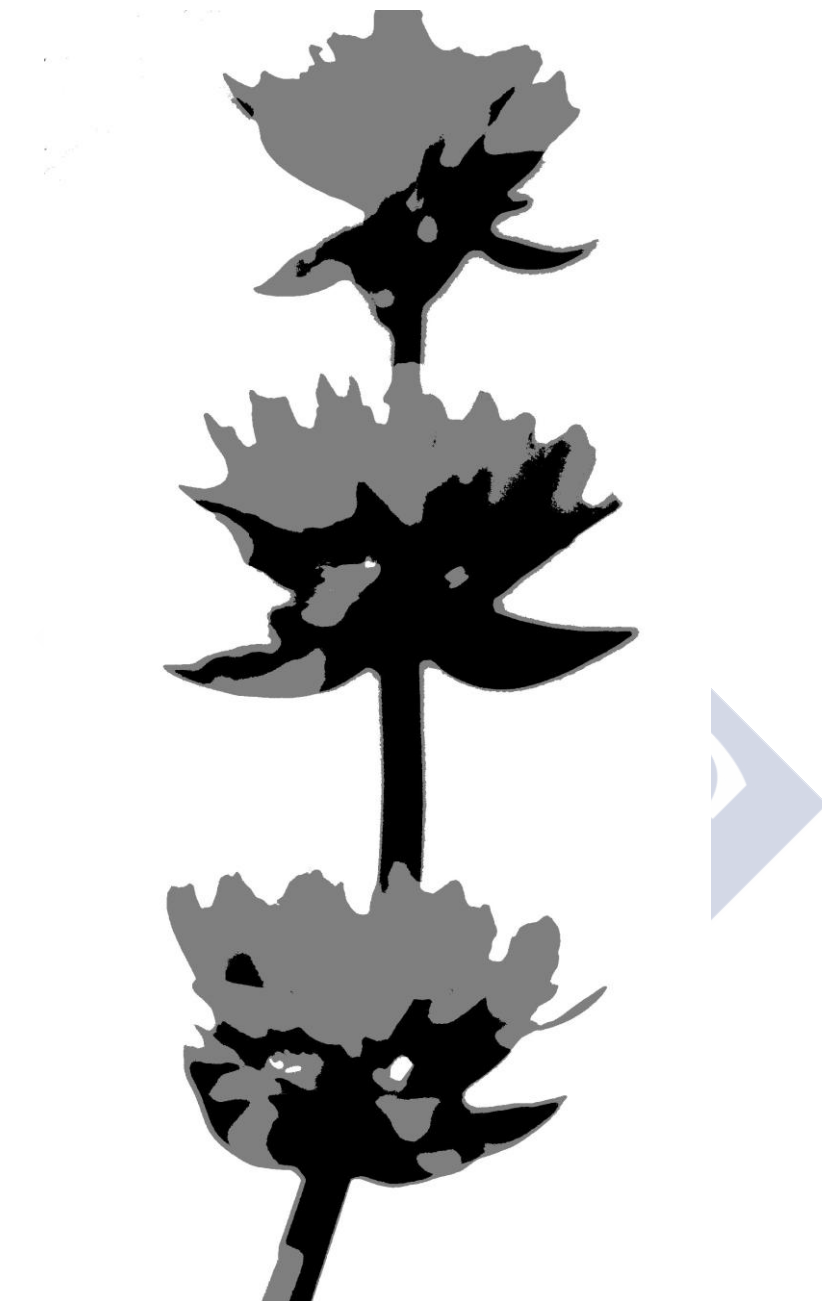
- Arista, M., M. Talavera, R. Berjano, and P. L. Ortiz. 2013. Abiotic factors may explain the geographical distribution of flower colour morphs and the maintenance of colour polymorphism in the scarlet pimpernel. *Journal of Ecology* 101:1613-1622.
- Armbruster, W. S. 2002. Can indirect selection and genetic context contribute to trait diversification? A transition-probability study of blossom-colour evolution in two genera. *Journal of Evolutionary Biology* 15:468-486.
- Bradshaw, H., and D. W. Schemske. 2003. Allele substitution at a flower colour locus produces a pollinator shift in monkeyflowers. *Nature* 426:176-178.
- Briscoe, A. D., and L. Chittka. 2001. The evolution of color vision in insects. *Annual Review of Entomology* 46:471-510.
- Campbell, D. R. 2009. Using phenotypic manipulations to study multivariate selection of floral trait associations. *Annals of Botany* 103:1557-1566.
- Carlson, J. E., and K. E. Holsinger. 2013. Direct and indirect selection on floral pigmentation by pollinators and seed predators in a color polymorphic South African shrub. *Oecologia* 171:905-919.
- Caruso, C. M., S. L. Scott, J. C. Wray, and C. A. Walsh. 2010. Pollinators, herbivores, and the maintenance of flower color variation: a case study with *Lobelia siphilitica*. *International Journal of Plant Science* 171:1020-1028.
- Chittka, L., and N. M. Waser. 1997. Why red flowers are not invisible to bees. *Israel Journal of Plant Sciences* 45:169-183.
- Eckhart, V. M. 1992. Spatio-temporal variation in abundance and variation in foraging behaviour of the pollinators of gynodioecious *Phacelia linearis* (Hydrophyllaceae). *Oikos* 64:573-586.
- Fenster, C. B., W. S. Armbruster, P. Wilson, M. R. Dudash, and D. James. 2004. Pollination syndromes and floral specialization. *Annual Review of Ecology, Evolution and Systematics* 35:375-403.
- Fineblum, W. L., and M. D. Rausher. 1997. Do floral pigmentation genes also influence resistance to enemies? The W locus in *Ipomoea purpurea*. *Ecology* 78:1646-1654.

- Frey, F. M. 2004. Opposing natural selection from herbivores and pathogens may maintain floral-color variation in *Claytonia virginica* (Portulacaceae). *Evolution* 58:2426-2437.
- Gómez, J. 2000. Phenotypic selection and response to selection in *Lobularia maritima*: importance of direct and correlational components of natural selection. *Journal of Evolutionary Biology* 13:689-699.
- Grace, J. B. 2006. Structural equation modeling and natural systems. Cambridge University Press, New York, USA.
- Green, B., and D. Durnford. 1996. The chlorophyll-carotenoid proteins of oxygenic photosynthesis. *Annual Review of Plant Biology* 47:685-714.
- Irwin, R. E., S. Y. Strauss, S. Storz, A. Emerson, and G. Guibert. 2003. The role of herbivores in the maintenance of a flower color polymorphism in wild radish. *Ecology* 84:1733-1743.
- Jones, K. M., and J. S. Reithel. 2001. Pollinator-mediated selection on a flower color polymorphism in experimental populations of *Antirrhinum* (Scrophulariaceae). *American Journal of Botany* 88:447-454.
- Kingsolver, J. G., H. E. Hoekstra, J. M. Hoekstra, D. Berrigan, S. N. Vignieri, C. E. Hill, A. Hoang, P. Gibert, and P. Beerli. 2001. The strength of phenotypic selection in natural populations. *The American Naturalist* 157:245-261.
- Kolb, A., J. Ehrlén, and O. Eriksson. 2007. Ecological and evolutionary consequences of spatial and temporal variation in pre-dispersal seed predation. *Perspectives in Plant Ecology, Evolution and Systematics* 9:79-100.
- Kugler, H. 1943. Hummeln als Blütenbesucher. *Ergebnisse der Biologie* 19:323.
- Kunze, J., and A. Gumbert. 2001. The combined effect of color and odor on flower choice behavior of bumble bees in flower mimicry systems. *Behavioral Ecology* 12:447-456.
- Lande, R., and S. J. Arnold. 1983. The measurement of selection on correlated characters. *Evolution* 37:1210-1226.
- Lev-Yadun, S., and K. S. Gould. 2009. Role of anthocyanins in plant defence. Pages 21-48 *In* Gould, K., K. Davies, and C. Winefield, eds. *Anthocyanins: Biosynthesis, Functions, and Applications*, Springer, New York, USA.
- Ma, J. F., P. R. Ryan, and E. Delhaize. 2001. Aluminium tolerance in plants and the complexing role of organic acids. *Trends in Plant Science* 6:273-278.
- Moehs, C. P., L. Tian, K. W. Osteryoung, and D. DellaPenna. 2001. Analysis of carotenoid biosynthetic gene expression during marigold petal development. *Plant Molecular Biology* 45:281-293.
- Moreno, J. M., F. D. Pineda, and S. Rivas-Martinez. 1990. Climate and vegetation at the Eurosiberian-Mediterranean boundary in the Iberian Peninsula. *Journal of Vegetation Science* 1:233-244.
- Peitsch, D., A. Fietz, H. Hertel, J. de Souza, D. F. Ventura, and R. Menzel. 1992. The spectral input systems of hymenopteran insects and their receptor-based colour vision. *Journal of Comparative Physiology A* 170:23-40.
- Ploquin, E. F., J. M. Herrera, and J. R. Obeso. 2013. Bumblebee community homogenization after uphill shifts in montane areas of northern Spain. *Oecologia* 173:1649-1660.

- Price, M. V., N. M. Waser, R. E. Irwin, D. R. Campbell, and A. K. Brody. 2005. Temporal and spatial variation in pollination of a montane herb: a seven-year study. *Ecology* 86:2106-2116.
- Rausher, M. D. 2008. Evolutionary transitions in floral color. *International Journal of Plant Sciences* 169:7-21.
- Rodríguez-Gironés, M. A., and L. Santamaría. 2004. Why are so many bird flowers red? *PLoS Biology* 2:e350.
- Rodríguez-Gironés, M. A., and L. Santamaría. 2005. Resource partitioning among flower visitors and evolution of nectar concealment in multi-species communities. *Proceedings Biological sciences / The Royal Society* 272:187-192.
- Rossi, M. 2011. Taxonomy, phylogeny and reproductive ecology of *Gentiana lutea* L. PhD dissertation. Università Degli Studi di Bologna; Italy.
- Schaefer, H. M., and G. Rolshausen. 2006. Plants on red alert: do insects pay attention? *BioEssays* 28:65-71.
- Schemske, D. W., and P. Bierzychudek. 2007. Spatial differentiation for flower color in the desert annual *Linanthus parryae*: was Wright right? *Evolution* 61:2528-2543.
- Spaethe, J., J. Tautz, and L. Chittka. 2001. Visual constraints in foraging bumblebees: flower size and color affect search time and flight behavior. *Proceedings of the National Academy of Sciences of the United States of America* 98:3898-3903.
- StatSoft, I. 2001. STATISTICA (data analysis software system), version 6. Tulsa, USA.
- Strauss, S. Y., and R. E. Irwin. 2004. Ecological and evolutionary consequences of multispecies plant-animal interactions. *Annual Review of Ecology, Evolution and Systematics* 35:435-466.
- Strauss, S. Y., R. E. Irwin, and V. M. Lambrix. 2004. Optimal defence theory and flower petal colour predict variation in the secondary chemistry of wild radish. *Journal of Ecology* 92:132-141.
- Strauss, S. Y., and J. B. Whittall. 2006. Non-pollinator agents of selection on floral traits. Pages 120-138 *In* Harder, L. D., and S. C. H. Barrett, eds. *Ecology and Evolution of Flowers*, Oxford University Press, New York.
- Streisfeld, M. A., and J. R. Kohn. 2007. Environment and pollinator-mediated selection on parapatric floral races of *Mimulus aurantiacus*. *Journal of Evolutionary Biology* 20:122-132.
- Tanaka, Y., and A. Ohmiya. 2008. Seeing is believing: engineering anthocyanin and carotenoid biosynthetic pathways. *Current Opinion in Biotechnology* 19:190-197.
- Tanaka, Y., N. Sasaki, and A. Ohmiya. 2008. Biosynthesis of plant pigments: anthocyanins, betalains and carotenoids. *The Plant Journal* 54:733-749.
- Veiga Blanco, T., L. Galetto, and I. Machado. 2013. Nectar regulation in *Euphorbia tithymaloides* L., a hummingbird-pollinated Euphorbiaceae. *Plant Biology* 15:910-918.
- Yoshida, K., Y. Toyama-Kato, K. Kameda, and T. Kondo. 2003. Sepal color variation of *Hydrangea macrophylla* and vacuolar pH measured with a proton-selective microelectrode. *Plant and Cell Physiology* 44:262-268.
- Zhu, C., S. Yamamura, H. Koiwa, M. Nishihara, and G. Sandmann. 2002. cDNA cloning and expression of carotenogenic genes during flower development in *Gentiana lutea*. *Plant Molecular Biology* 48:277-285.

- Zhu, C., S. Yamamura, M. Nishihara, H. Koiwa, and G. Sandmann. 2003. cDNAs for the synthesis of cyclic carotenoids in petals of *Gentiana lutea* and their regulation during flower development. *Biochimica et Biophysica Acta (BBA)-Gene Structure and Expression* 1625:305-308.
- Zhu, C., C. Bai, G. Sanahuja, D. Yuan, G. Farré, S. Naqvi, L. Shi, T. Capell, and P. Christou. 2010. The regulation of carotenoid pigmentation in flowers. *Archives of Biochemistry and Biophysics* 504:132-141.





5. SELECTIVE PRESSURES
EXPLAIN DIFFERENCES IN
FLOWER COLOR AMONG
Gentiana lutea L. POPULATIONS



5.1 ABSTRACT

Flower color variation among plant populations might reflect adaptation to local conditions such as the interacting animal community. At the northwest of the Iberian Peninsula, flower color of *Gentiana lutea* varies among populations from orange to yellow with a longitudinal pattern. We inspected the variation in selective pressures on flower color among 12 *G. lutea* populations (by analyzing the variation in fitness-color relationships), and we tested whether selective pressures on flower color (total selection differentials) explain color variation along the longitudinal range studied. We additionally assessed the possible role of pollinators and seed predators on this selection by analyzing (i) the influence of flower color on pollinator visitation rate and (ii) escape from seed predation and (iii) the relationships between flower color variation and the pollinator community. We found that selective pressures explain part of the flower color differences among populations in *G. lutea* and pollinators (but not seed predators) play a role on flower color local adaptation.

5.2 INTRODUCTION

Variation in flower color among plant populations may reflect adaptation to local selective pressures (Streisfeld and Kohn 2005, Rausher 2008, Arista et al. 2013). Among distant populations, we expect low gene flow and high variation in the biotic and abiotic environments. Differences in natural selection among populations may occur due, for example, to the spatial variation in the interacting animal community (Thompson 1994). Most of the studies that evaluate the adaptive nature of flower color, at the intraspecific level, focus on color variation within populations, rather than among populations (Rausher 2008). However, to solve the question of whether flower color variation reflects local adaptation, it is required to focus on flower color variation among populations: we may determine on the one hand whether color is an adaptive trait and on the other hand if color differences among populations arise from locally different selective pressures.

Since Darwin seminal studies, floral variation called the attention of evolutionary biologists due to the role of flowers as plant's reproductive organs susceptible to be selected by pollinators. Among floral traits, flower color is one of the most recognized attribute to influence pollinators (Rausher 2008) —flower color defines pollination syndromes linked to pollinator groups such as hummingbirds, bees or bats (Fenster et al. 2004). However, non-pollinator (biotic or abiotic) agents may also influence species diversification through flower color, and thus color variation may result from several selective agents (reviewed in Strauss and Whittall 2006 and in Rausher 2008, Caruso et al. 2010). There is growing evidence of the importance of non-pollinator selection on flower color (Irwin et al. 2003, Frey 2004, Strauss et al. 2004, Strauss and Whittall 2006, Carlson and Holsinger 2013), but few studies have explored selection exerted by predispersal seed predators (Kolb et al. 2007). Predispersal seed predators are agents of selection at least within a *G.lutea* population studied (see chapter 4): adults oviposit during blooming (thus flower color may affect their choices) and larvae predate seeds, therefore affecting seed production (Kolb et al. 2007).

Gentiana lutea is a montane species which typically shows yellow corollas all over its distribution; though, at the southwestern end of its range (southwest Europe, Iberian Peninsula, from approximately 5° 30' W, to the west) the species blooms with orange flowers. Corolla color in *Gentiana lutea* has genetic basis: flower pigmentation depends

upon the quantity and content of carotenoids (Zhu et al. 2002 and 2003), which is regulated by genes that control their synthesis and storage (Tanaka et al. 2008, Zhu et al. 2010). Flower color variation within a population of this species affects plant reproductive success through the action of pollinators and seed predators (see chapter 4), which may be also selective agents influencing color variation among populations. In this chapter we tested if local selective pressures explain flower color variation among *G. lutea* populations (rather than aiming to find selection on flower color within populations, which was the aim in chapter 4). In addition, we studied the possible role of pollinators and seed predators as selective agents driving color variation among populations. We expect spatial variation in the selective pressures throughout a longitudinal range of 230 km, in which *G. lutea* flower color varies (Fig. 3.2 in chapter 3, page 47); these different selection scenarios might affect variation in flower color among populations. Besides, we presume that pollinators and seed predators are selective agents influencing flower color variation among populations.

Demonstrating variation in selective pressures among different populations and analyzing its effect on the spatial variation of plant traits among populations is a powerful tool to measure the effect of natural selection in the wild (see Herrera et al. 2006, Rausher 2008, Alcántara et al. 2010). Therefore, we (1) analyzed the effect of flower color on fitness and the spatial heterogeneity of this effect (otherwise, we assess the spatial variation in selective pressures). But, detecting variation in selective pressures among populations does not demonstrate that natural selection drives phenotypic variation; to provide evidence of local adaptation it is necessary to show that local selective pressures explain, at least in part, the phenotypic variation among populations (Herrera et al. 2006). Therefore, we secondly tested (2) whether the selective pressures explain flower color variation among populations. Finally, we were interested in finding if mutualist pollinators and antagonist seed predators play a role in flower color adaptation among populations —such as we found within a *G. lutea* population in chapter 4. To test the role of pollinators and predators as selective agents we concretely studied (3) the interaction of plants with pollinators and seed predators depending on their flower color across populations, and (4) the differences in flower color variation among populations related to pollinator communities. We show that natural selection, exerted likely through pollinators but no seed predators, influence flower color variation among populations in *Gentiana lutea*.

5.3 MATERIALS AND METHODS

5.3.1 Field procedures

We studied 12 *G. lutea* populations across two different years (nine in 2010 and 12 in 2011). In order to properly test the spatial variation of selection we focused our sampling effort in the number of populations and the geographical range covered. The study area covered the western end of the species distribution in the Cantabrian Mountains. Populations were chosen haphazardly along a longitudinal transect from San Mamede population (7° 30' W, west) until San Glorio (4° 45' W, east), 230 km apart (Fig. 2.2 in chapter 2, page 29). Longitudinal coordinates were obtained for each population with a GPS (Garmin eTrex Vista).

In June, when the stem was developed but before blooming started, we haphazardly chose in each population between 20 and 45 plants each year. We aimed for a larger number of plants within each population to be able to detect significant selection differentials of small magnitude (see Alcántara et al. 2010). However, duration of blooming in *Gentiana lutea* is reduced to several days within each population and thus

it was not possible to increase the number of plants studied within populations while maintaining the number of populations studied. Besides, the number of flowering plant individuals in some populations was small, thus increasing sample size was not possible.

5.3.1.1 Flower color and other plant traits

In July, during blooming, we measured flower color on ten randomly chosen flowers per plant in 2010. We measured color on three flowers per plant in 2011 because the coefficient of variation for flower color within plants was asymptotic after 3 flowers. Details on the methodology to measure flower color and to transform the spectra on color variables is already described in section 2.3, chapter 2. We measured floral color in a total of 2711 flowers belonging to 504 plants across 12 *G. lutea* populations. Besides floral color, we additionally measured in each plant: petal length (mm), petal width (mm), leaf length (the length of the longest basal leave, mm), and we counted the number of flowers. Petal length and petal width were obtained from three petals per plant belonging to three randomly chosen flowers and averaged. We measured petal length from the insertion until the tip and petal width on the mid part (the widest); we used a caliper to the nearest 0.1 mm. We measured the length of the longest basal leave from the insertion to the tip; with a measuring tape to the nearest 1 mm.

5.3.1.2 Plants pollinator visitation rate

We determined the identity and abundance of pollinators for each plant by counting all insects visiting flowers and making contact with the anthers and stigmas. We made 10 censuses of 1 minute per plant during 2010 and 10 censuses of 2 minutes per plant in 2011; a total of 130 h of censuses to 466 plants. Censuses were done between 10:00 h and 19:00 h (Greenwich Mean Time) with temperatures ranging between 12° C and 26° C, no windy conditions or rain. We considered pollinator visitation rate as the number of total visits per minute for each plant. We captured some specimens to further identification in the laboratory, but some pollinator species were difficult to discern during census; thus we assessed the pollinator community using distinguishable pollinator groups. Due to resource limitation, we could not make censuses of seed predators or culture of larvae; thus, we lack information on seed predator community.

5.3.1.3 Plants escape from seed predation

In August both years, when fruits were ripen but not yet opened, we counted the total number of fruits and we examined seed predation in each of 431 plants. From ten randomly selected fruits in each plant, we counted the number of fruits affected by seed predators (fruits showed unequivocal signals such as holes or rotting, thus it was unnecessary to open them). We obtained the rate of the escape from seed predation as the percentage of fruits that were not affected by predators.

5.3.1.4 Plants seed production

We collected between seven and ten fruits per plant and counted the number of seeds within them. We estimated the total seed production of each plant as the mean seeds per fruit, multiplied by the total number of fruits of each plant. We examined seed production for 3644 fruits belonging to 383 plants. We used seed production per plant as a proxy for fitness; in long-lived species (such as *G. lutea*) seed production—and other measures of reproductive success—are accepted surrogates of fitness since measuring total fitness is often not possible (see Kingsolver et al. 2001).

5.3.2 Statistical analysis

Although we focus on flower color, we include other phenotypic traits in the following analyses; those traits might influence pollination, seed predation and seed production, and might correlate with flower color (some floral traits and color use to covary and in fact are used to define the floral syndromes, see Fenster et al. 2004). By including other phenotypic traits, we control for their effects.

We follow the approach developed by Herrera et al. (2006) to provide evidence of natural selection driving local adaptation among populations: we analyzed (1) whether the selective pressures on flower color vary among populations and if (2) those selective pressures affect the spatial variation of flower color among populations.

To show whether pollinators and seed predators play a role on the local adaptation, influencing color variation among populations, we assessed if (3) the interaction of plants with pollinators and seed predators depended on flower color in a different manner between populations and if (4) differences among populations in flower color are related to variation in pollinator communities.

5.3.2.1 Variation of selective pressures among populations

To address the spatial variation in selective pressures we analyzed the variation among populations in the relationship of plant traits and a measure of fitness—following the procedure indicated in Herrera et al. (2006). In this step, we aim to test for differences among populations in natural selection, not to measure selection within populations. We built a generalized linear model (GzLM) model in which seed production was the dependent variable. We considered the number of seeds produced by each plant as a proxy for plant fitness, which is an accepted surrogate in long-lived species (Kingsolver et al. 2001). Independent variables included: population, year, plant traits (flower color, petal width, petal length, leaf length and number of flowers), and two-way interactions between population and each plant trait. We included population as a fixed factor because we are interested in the variation of selective pressures among populations (population * trait interactions). The models were selected following AICc criterion (Quinn and Keough 2002): seed production was fitted to a gamma distribution with a logarithmic link function.

5.3.2.2 Effect of selective pressures on the spatial variation of flower color

We aim to test whether selective pressures exerted on flower color explain flower color variation among populations in *Gentiana lutea*, rather than measuring selection on color within populations (although we calculated the coefficients for total, direct, quadratic and correlational selection, and we detected significant selection coefficients on flower color on eight of the twelve populations; Table A1). To this purpose, we must first calculate the selection differentials (S) on flower color, to further use them as the independent variable in the model explaining flower color variation among populations. The selection differential on flower color is the standardized coefficient of a simple regression of relative fitness on flower color (Lande and Arnold 1983, Conner and Hartl 2004), for each population and year. The selection differential estimates the strength of total selection exerted by all selecting forces. In this case, selection differentials account for both the positive effect of mutualistic pollinators and the negative effect of antagonistic seed predators. Additionally, selection differentials include the effect of selection exerted by other selective forces and selection exerted throughout correlated traits (indirect selection). Since in here we want to evaluate the total outcome of selection (direct and indirect) on flower color; we use the total selection differentials, instead of selection gradients that only estimate direct selection. We calculated the

relative fitness for each plant, dividing its seed production by the mean seed production in each population, each year.

We designed a generalized linear mixed model (GLIMMIX) with the following explanatory variables: the selection differentials on flower color (S), the geographical longitude, plant traits (petal width, petal length, leaf length and number of flowers) and year, as fixed factors; and population as a random factor. Plant traits are measured at the plant-level whereas the selection differential and the geographical coordinate are measured at the population-level. We included population as a random factor that nested plants within populations, thus we obtain adequate degrees of freedom to correctly estimate the level of significance and parameters of the explanatory variables (we followed a similar procedure as in Sobral et al. 2013). The effects of the selection differential and the geographical coordinate will relate to population's mean flower color, not to plant individuals flower color. Note that we run an equivalent model using populations' averages instead of plant-level information and we obtained equivalent results (results not shown). We used satterthwaite's method to calculate the degrees of freedom. The model was selected following the AICc criterion (Quinn and Keough 2002): flower color fitted a normal distribution with a logarithmic link function. We calculated also the proportion of variance explained by each factor by running an univariate analysis of variance with flower color as the response variable (log-transformed) and the statistical significant factors as the explanatory factors. The proportion of variance was calculated from the type III sum of squares.

5.3.2.3 Flower color effect on pollination and seed predation; among population variation

To investigate the spatial variation in the effect of flower color on pollination and escape from seed predation, we performed two GzLMs, one for pollinator visitation rate and the other for escape from seed predation as dependent variables. In these models, plant traits analyzed were flower color, petal width, petal length, leaf length and number of flowers. We included also population, year, and two-way interactions between population and plant traits. The interactions between traits and populations indicate if preferences vary among populations. We considered population as a fixed factor because we focus on differences among populations. The models were selected following AICc criterion (Quinn and Keough 2002): pollinator visitation rate was fitted to a gamma distribution with a logarithmic link function; escape from seed predation was fitted to a poisson distribution with a logarithmic link function. Plant traits were centered (we subtracted the average) to avoid collinearity (Quinn and Keough 2002).

All previous statistical analyses were performed with the SPSS for windows version 20 (IBM SPSS Statistics 20; IBM Corp., Somers, NY).

5.3.2.4 Flower color and pollinator community

We accounted for the spatial variation in the pollinator community and its relationship with flower color variation among populations. We built a dissimilarity matrix on the pollinator assemblage following the procedures described in Manzaneda and Rey (2008) for each population and year. We calculated also one matrix with flower color differences between populations (the absolute differences between color population means). We tested the correlation between both matrices (eight populations in 2010 and 12 populations in 2011, which results in 190 comparisons) by the Mantel test (Manteltester 1.0; Bonnet and Van de Peer 2002).

5.4 RESULTS

Flower color is correlated with petal width ($r = -0.493$, $P = 0.000$, $N = 429$) and with the number of flowers ($r = 0.166$, $P = 0.001$, $N = 429$): orange plants have wider petals and fewer flowers when compared to the yellow ones. We detected significant and positive correlations among leaf length and all the floral attributes measured: petal width ($r = 0.115$, $P = 0.017$, $N = 429$), petal length ($r = 0.270$, $P = 0.000$, $N = 429$) and the number of flowers ($r = 0.230$, $P = 0.000$, $N = 429$). In addition, petal width correlates positively with petal length ($r = 0.524$, $P = 0.000$, $N = 429$) and negatively with the number of flowers ($r = -0.140$, $P = 0.004$, $N = 429$).

5.4.1 Variation of selective pressures among populations

Overall, flower color influenced seed production. But, selective pressures on flower color varied among populations: we detected spatial variation in the relationship between flower color and seed production (significant flower color*population interaction). The number of flowers also affected seed production, but its effect did not vary among populations (Table 5.1).

5.4.2 Effect of selective pressures on the spatial variation of flower color

Selection differentials (total selection) explained part of the flower color variation among populations: plants growing in populations where selective pressures favor yellowness, bloom with yellow flowers; plants growing in populations where selective pressures favored the orange coloration, tended to bloom with orange coloration (Table 2.2; Fig. 5.1). The longitudinal coordinate explained part of the flower color variation among populations: yellowness decreases westward (Table 5.2): flower color differences among populations are explained mostly by the geographical longitude (45 %), followed by the selection differential which explains 6% of the variation in flower color among populations.

Table 5.1. Results of the GzLM fitted to analyze the variation among populations in the selective pressures on flower color (variations in the relationship between flower color of plant individuals and plant fitness) N = 350. In bold are effects with P values < 0.05. Factor codes: PL, petal length; PW, petal width; FN, number of flowers; LL, leaf length; Pop, population.

Dependent variable	Factor	Coefficient estimate	s.e	Statistic	d.f.	P
Seed production	Flower color	0.100	0.227	4.168	1	0.041
	PL	0.064	0.098	2.535	1	0.111
	PW	0.065	0.461	0.390	1	0.532
	FN	0.008	0.007	23.971	1	0.000
	LL	0.001	0.004	2.327	1	0.127
	Pop			117.049	11	0.000
	Year			2.009	1	0.156
	Pop * year			4.475	7	0.724
	Pop * flower color			21.120	11	0.032
	Pop * PL			8.446	11	0.673
	Pop * PW			7.483	11	0.759
	Pop * FN			7.865	11	0.725
	Pop * LL			15.817	11	0.148

Table 5.2. Results of the GLIMMIX model fitted to analyze the effect that total selection coefficients have on flower color variation among populations of *G. lutea*. For fixed effects, the statistic is F and for random factors, the statistic is Z. N = 410. In bold are effects with P values < 0.05. We calculated the variance explained by each variable from the type I sum of squares; the proportion of variance not explained by the factors (the error) is 44%.

Dependent variable	Factor	Coefficient estimate	s.e	Statistic	P
Flower color	Total selection coefficient (S)	-0.201	0.096	4.344	0.038
	Longitudinal coordinate	-0.332	0.073	20.801	0.002
	Flower number	0.000	0.000	0.245	0.621
	Petal width	0.027	0.015	3.374	0.067
	Petal length	-0.002	0.004	0.342	0.559
	Leaf length	0.000	0.000	0.017	0.896
	Year			4.158	0.042
	Population			1.815	0.069

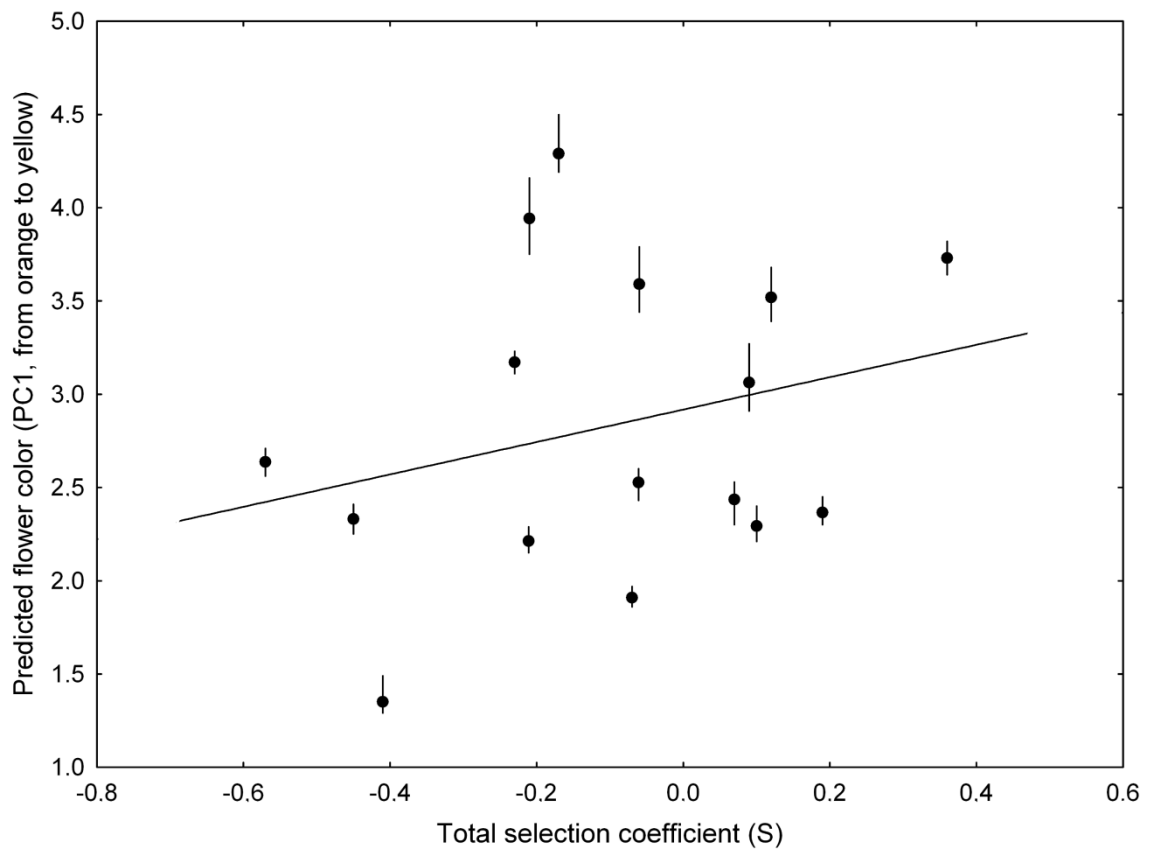


Figure 5.1. Relationship between total selection coefficients on flower color and flower color variation among populations. Dots represent estimated population-level means and bars show their minimum and maximum. We studied 12 populations across two different years (six populations were studied across two years and other six populations were studied one year, therefore we have 18 data points).

5.4.3 Flower color effect on pollination and seed predation; among population variation

The mean number of pollinator visits per minute per plant was 1.24 ($\pm$ 1.54 s.d.); we recorded a total of 7016 pollinator visits to 466 plants during 130 h of censuses. Mean escape from seed predation rates per plant averaged 65.2% $\pm$ 31.7% s.d. Flower color affected pollinator's visits and this effect varied among populations. We did not detect an effect of flower color on seed predation. Moreover, petal length and number of flowers also affected pollinator visitation rate and escape from seed predation, but we did not detect any spatial variation of the effect of those traits (Table 5.3).

Table 5.3. Results of the GzLMs fitted to analyze the variation among populations in the influence of flower color on both pollinator visitation rate and escape from seed predation in *G. lutea*. For pollinator visitation rate, N = 443; for escape from seed predation, N = 406. In bold are effects with P values < 0.05. Factor codes: PL, petal length; PW, petal width; LL, leaf length; FN, number of flowers; Pop, population.

Dependent			Factor		Coefficient		Chi-square	d.f.	P
					estimate	s.e			
Pollinator visitation rate	Flower color				0.249	0.187	0.009	1	0.925
	PL				0.235	0.084	7.987	1	0.005
	PW				-0.091	0.305	1.238	1	0.267
	FN				-0.007	0.006	8.174	1	0.004
	LL				0.004	0.004	2.225	1	0.136
	Pop						123.756	11	0.000
	Year						16.559	1	0.000
	Pop * year						43.647	8	0.000
	Pop * flower color						25.000	11	0.009
	Pop * PL						9.055	11	0.617
	Pop * PW						11.351	11	0.414
	Pop * FN						11.495	11	0.403
	Pop * LL						11.263	11	0.421
Escape from seed predation	Flower color				0.016	0.105	0.004	1	0.950
	PL				0.051	0.048	7.374	1	0.007
	PW				-0.176	0.196	2.503	1	0.114
	FN				-0.003	0.003	5.330	1	0.021
	LL				0.000	0.002	1.176	1	0.278
	Pop						65.451	11	0.000
	Year						2.786	1	0.095
	Pop * year						77.389	8	0.000
	Pop * flower color						8.086	11	0.706
	Pop * PL						13.011	11	0.293
	Pop * PW						11.317	11	0.417
	Pop * FN						15.071	11	0.179
	Pop * LL						7.336	11	0.771

5.4.4 Flower color and pollinator community

Gentiana lutea plants received visits from bumblebees (79%), cuckoo bumblebees (20%), and honeybees (1%). Within bumblebees we found ten species: *B. terrestris*, *B. lucorum*, *B. pratorum*, *B. soroeensis ancaricus*, *B. lapidarius*, *B. wurflenii*, *B. hortorum*, *B. jonellus*, *B. mesomelas*, and *B. pascuorum* (Table A2). Differences in pollinator communities correlate to differences in flower color among populations (8 populations in 2010, 12 populations in 2011; $N = 190$, $r = 0.208$, $P < 0.05$; Fig. 5.2).

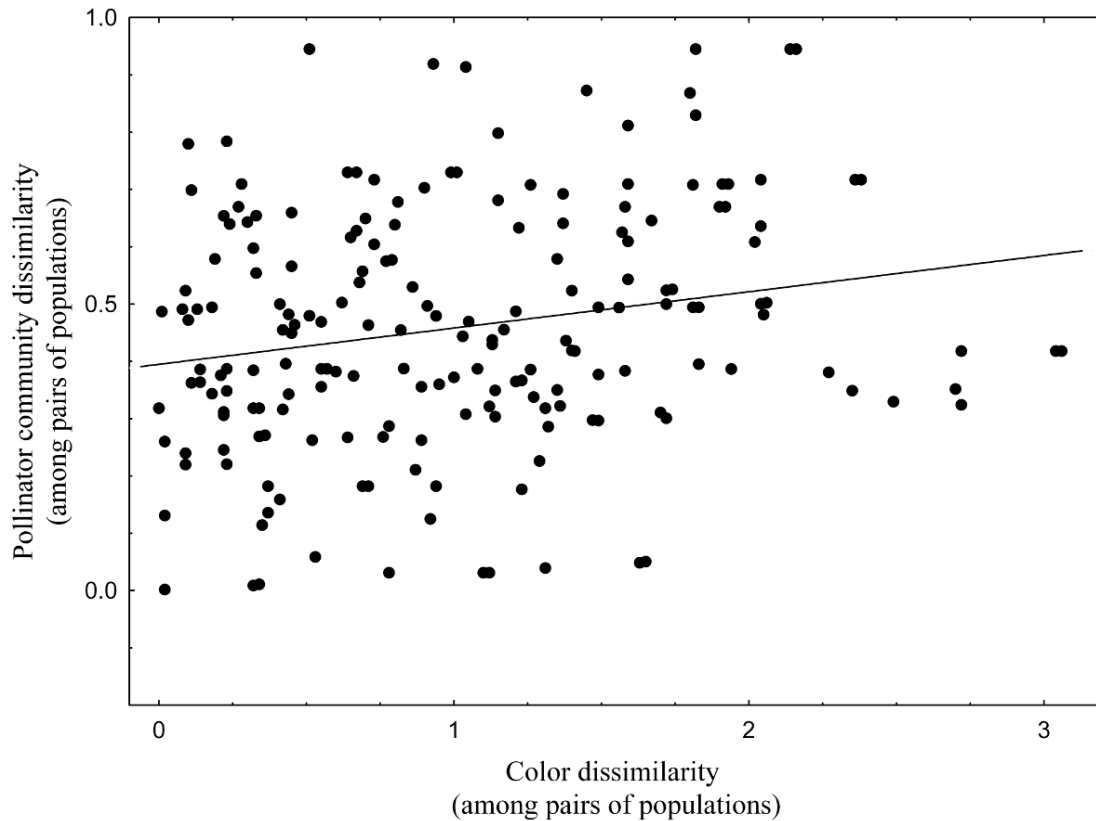


Figure 5.2. Pollinator community and color variation. Relationship between flower color dissimilarity among populations and pollinator community dissimilarity among populations. We built a dissimilarity matrix on the pollinator assemblage following the procedures described in Manzaneda and Rey (2008) for each population and year. We calculated also one matrix with flower color differences between populations (the absolute differences between color population means). 8 populations in 2010, 12 populations in 2011; $N = 190$, $r = 0.208$, $P < 0.05$.

5.5 DISCUSSION

Our results support the hypothesis that natural selection drives, at some extent, the geographic structure of flower color variation among populations in this species, most likely through the action of pollinators. We base our conclusion (provided that corolla color has genetic basis; Zhu et al. 2002 and 2003) in five facts: (1) flower color influenced seed production and the selective pressures on flower color differed among populations, (2) total selection differentials on flower color within each population explained part of the variation in flower color among populations, (3) flower color affects pollinator visitation rate and this effect is different between populations and (4) differences in the composition of pollinator assemblage correlate with flower color

differences among populations. Despite the well studied relationship between pollinators and floral variation, only a few studies provided evidence about the role of pollinators in the geographical structure of flower color variation (Streisfeld and Kohn 2007, Newman et al. 2012). We studied also the role of seed predators as selective agents and our results show that they are not selective agents driving color variation among populations; to our knowledge, this is the first study regarding the role of non-pollinator agents in the geographical structure of flower color variation.

To demonstrate that natural selection explains flower color variation among populations, it is necessary to consider the selection differentials which measure total selection (direct and indirect), rather than selection gradients which account only for direct selection (see equivalent approaches in Herrera et al. 2006, Sobral et al. 2013). The selection differential accounts for overall selection: the mutualistic effect of pollinators, the antagonistic effect of seed predators, other selective forces that may act on flower color, and also the indirect effect of selection through correlated characters. Showing that flower color varies among populations of *G. lutea*, and that this variation is explained by local selective pressures, while identifying the agents of selection, increases our understanding of the adaptive nature of flower color.

Differences in the pollinator community are correlated with flower color variation among populations of *Gentiana lutea*. Pollinator species might have dissimilar preferences towards floral traits, such as color: the visual system of pollinators differs among species (Chittka and Waser 1997, Briscoe and Chittka 2001), which causes different ability to detect particular colors (Rodríguez-Gironés and Santamaría 2004). Pollinators may exert selection on color because they may prefer the colors that decrease their searching time, which translates into a higher foraging efficiency (provided that other factors such as abundance or quality of resources are similar among colors). In here, we support the hypothesis that differences among populations in selective pressures on flower color are related to differences in the pollinator communities: the species *Bombus terrestris* manifests low sensitivity for detecting red colors (Peitsch et al. 1992, Briscoe and Chittka 2001) and is more abundant in yellow-flowered populations than in orange-flowered populations (Table A2). Additionally, *Bombus lapidarius* shows sensitivity for red signals (although it lacks a red color receptor; Kugler 1943, Peitsch et al. 1992), and it is abundant in some orange-flowered populations and scarce in yellow-flowered populations (Table A2). Thus, variation in the community composition may cause different selective pressures among populations and affect flower color variation among populations of the same plant species.

Seed predators are not selective agents driving color variation among populations in *G. lutea* since we did not detect an effect of flower color on seed predation. However, seed predators may share targets with pollinators (the most pollinated plants will produce more seeds, which are food resources) and thus they may be selective agents on other floral traits. In *G. lutea* petal length and the number of flowers affect both pollinators and predators, thus seed predators may exert selection on those traits. In addition, since the number of flowers correlate to flower color (Table 5.2), flower color may be subject to indirect selection by seed predators, so we may not completely discard their role as selective agents on color. In fact, we demonstrated the role of seed predators as selective agents on flower color within a population (chapter 4).

Apart from the spatial variation of selection driven by the animal community, other non-mutually exclusive hypotheses might also explain the geographic structure of flower color in this species. These hypotheses include for example selective pressures exerted by abiotic conditions such as climate or soil properties, plasticity in flower color responding to those abiotic conditions, historical factors and genetic drift.

Abiotic factors could in theory exert influence on color diversification. Genes responsible for pigments may also influence plant physiological traits that confer advantages to some environmental conditions (Strauss and Whittall 2006, Schemske and Bierzychudek 2007). Thus, the spatial variation of abiotic factors across the Cantabrian Range may contribute to the geographical structure of flower color variation. Climatic variation might be relevant: following the Köppen climate classification for the Iberian Peninsula (AEMET-IM 2011), we find climatic differences between the orange and the yellow flowering populations. Basically, the orange populations grow in a drier and warmer climate than the yellow populations. Additionally, those climatic conditions may influence the biotic community which in turn may affect flower color variation among populations. Even more, we explicitly analyzed the relationship of soil pH with color (see chapter 2, section 2.5, pages 36-37), and population temperature, radiation and precipitation with floral and vegetative traits (chapter 3) in this species and we have not found any relationship between these abiotic factors and *G. lutea* traits. Evidence suggests that selection by abiotic factors does not explain local adaptation among *G. lutea* populations.

Soil characteristics may affect flower color: pigments may vary as a plastic response to soil conditions (Ma et al. 2001, Yoshida et al. 2003, Borghesi et al. 2011) or due to local adaptation to soil properties (Schemske and Bierzychudek 2007). Moreno-Saiz et al. (2013) have recently proposed a biogeographical map of vascular flora of the Iberian Peninsula with two main regions: the east area with basic soils and the west area with acid soils and a high influence of the Atlantic Ocean. This pattern of variation might coincide with flower color at the population level in *G. lutea*. But, we found flower color varying from orange to yellow among plants within populations where climatic conditions and soil properties were similar. Besides this, we found yellow flowering populations growing on both basic and acid soils.

Historical events or genetic drift may affect the geographical patterns of color variation (Fenster et al. 2004). Genetic isolation and diversification of montane plant species in the Iberian mountains occurred due to the cyclical climatic changes in the Quaternary: populations rise in altitude and become isolated during warming in the post-glacial periods and spread into valleys during cold periods (Stewart et al. 2010, Alarcón et al. 2012). Those events might also influence the evolutionary dynamics in *Gentiana lutea* populations and explain the flower color variation across the Cantabrian Range.

Our aim was to address if selective pressures explain the geographic structure of color variation. Our results show that selective pressures explain the geographical structure of color variation (the selection differentials explain part of color variation among populations). Additionally, pollinators exert selection on color—in a different manner between populations—and differences in the pollinator communities are correlated to color differences among populations; we argue that pollinators are likely

selective forces influencing total selection on flower color and driving part of the flower color variation among populations. Assessing the impact of selection on phenotypic variation among populations is a powerful approach to identify local adaptation in natural conditions, especially when studying long-lived organisms throughout broad geographical scales (Herrera et al. 2006, Alcántara et al. 2010). In here, we show that natural selection—most likely exerted by pollinators—explains flower color variation among populations of *Gentiana lutea*.

5.6 REFERENCES

- AEMET-IM. 2011. Iberian Climate Atlas—Air temperature and precipitation (1971-2000). Agencia Estatal de Meteorología, Ministerio de Medio Ambiente y Medio Rural y Marino e Instituto de Meteorologia de Portugal (eds). ISBN: 978-84-7837-079-5.
- Alarcón, M., P. Vargas, L. Sáez, J. Molero, and J. J. Aldasoro. 2012. Genetic diversity of mountain plants: Two migration episodes of Mediterranean *Erodium* (Geraniaceae). *Molecular Phylogenetics and Evolution* 63:866-876.
- Alcántara, J., J. Bastida, and P. Rey. 2010. Linking divergent selection on vegetative traits to environmental variation and phenotypic diversification in the Iberian columbines (*Aquilegia*). *Journal of Evolutionary Biology* 23:1218-1233.
- Arista, M., M. Talavera, R. Berjano, and P. L. Ortiz. 2013. Abiotic factors may explain the geographical distribution of flower colour morphs and the maintenance of colour polymorphism in the scarlet pimpernel. *Journal of Ecology* 101:1613-1622.
- Bonnet, E., and Y. Van de Peer. 2002. zt: a software tool for simple and partial Mantel tests. *Journal of Statistical Software* 7:1-12.
- Borghesi, E., M. L. González-Miret, M. L. Escudero-Gilete, F. Malorgio, F. J. Heredia, and A. J. Meléndez-Martínez. 2011. Effects of salinity stress on carotenoids, anthocyanins, and color of diverse tomato genotypes. *Journal of Agricultural and Food Chemistry* 59:11676-11682.
- Briscoe, A. D., and L. Chittka. 2001. The evolution of color vision in insects. *Annual Review of Entomology* 46:471-510.
- Carlson, J. E., and K. E. Holsinger. 2013. Direct and indirect selection on floral pigmentation by pollinators and seed predators in a color polymorphic South African shrub. *Oecologia* 171:905-919.
- Caruso, C. M., S. L. Scott, J. C. Wray, and C. A. Walsh. 2010. Pollinators, herbivores, and the maintenance of flower color variation: a case study with *Lobelia siphilitica*. *International Journal of Plant Science* 171:1020-1028.
- Chittka, L., and N. M. Waser. 1997. Why red flowers are not invisible to bees. *Israel Journal of Plant Sciences* 45:169-183.
- Conner, J. K., and D. L. Hartl. 2004. A primer of ecological genetics. Sinauer Associates Incorporated, Sunderland.
- Fenster, C. B., W. S. Armbruster, P. Wilson, M. R. Dudash, and D. James. 2004. Pollination syndromes and floral specialization. *Annual Review of Ecology, Evolution and Systematics* 35:375-403.
- Frey, F. M. 2004. Opposing natural selection from herbivores and pathogens may maintain floral-color variation in *Claytonia virginica* (Portulacaceae). *Evolution* 58:2426-2437.

- Herrera, C. M., M. C. Castellanos, and M. Medrano. 2006. Geographical context of floral evolution: towards an improved research programme in floral diversification. Pages 278-294 *In* Harder, L. D., and S. C. H. Barret, eds. Ecology and evolution of flowers, Oxford University Press, Oxford, United Kingdom.
- IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp.
- Irwin, R. E., S. Y. Strauss, S. Storz, A. Emerson, and G. Guibert. 2003. The role of herbivores in the maintenance of a flower color polymorphism in wild radish. *Ecology* 84:1733-1743.
- Kingsolver, J. G., H. E. Hoekstra, J. M. Hoekstra, D. Berrigan, S. N. Vignieri, C. E. Hill, A. Hoang, P. Gibert, and P. Beerli. 2001. The strength of phenotypic selection in natural populations. *The American Naturalist* 157:245-261.
- Kolb, A., J. Ehrlén, and O. Eriksson. 2007. Ecological and evolutionary consequences of spatial and temporal variation in pre-dispersal seed predation. *Perspectives in Plant Ecology, Evolution and Systematics* 9:79-100.
- Lande, R., and S. J. Arnold. 1983. The measurement of selection on correlated characters. *Evolution* 37:1210-1226.
- Ma, J. F., P. R. Ryan, and E. Delhaize. 2001. Aluminium tolerance in plants and the complexing role of organic acids. *Trends in Plant Science* 6:273-278.
- Manzaneda, A. J., and P. J. Rey. 2008. Geographic variation in seed removal of a myrmecochorous herb: influence of variation in functional guild and species composition of the disperser assemblage through spatial and temporal scales. *Ecography* 31:583-591.
- Moreno-Saiz, J. C., M. Donato, L. Katinas, J. V. Crisci, and P. Posadas. 2013. New insights into the biogeography of south-western Europe: spatial patterns from vascular plants using cluster analysis and parsimony. *Journal of Biogeography* 40:90-104.
- Newman, E., B. Anderson, and S. D. Johnson. 2012. Flower colour adaptation in a mimetic orchid. *Proceedings Biological Sciences / The Royal Society* 279:2309-2313.
- Peitsch, D., A. Fietz, H. Hertel, J. de Souza, D. F. Ventura, and R. Menzel. 1992. The spectral input systems of hymenopteran insects and their receptor-based colour vision. *Journal of Comparative Physiology A* 170:23-40.
- Quinn, G. P., and M. J. Keough. 2002. *Experimental Design and Data Analysis for Biologists*. Cambridge University Press, Cambridge, United Kingdom.
- Rausher, M. D. 2008. Evolutionary transitions in floral color. *International Journal of Plant Sciences* 169:7-21.
- Schemske, D. W., and P. Bierzychudek. 2007. Spatial differentiation for flower color in the desert annual *Linanthus parryae*: was Wright right? *Evolution* 61:2528-2543.
- Sobral, M., J. Guitián, P. Guitián, and A. R. Larrinaga. 2013. Selective pressure along a latitudinal gradient affects subindividual variation in plants. *PloS one* 8:e74356.
- Stewart, J. R., A. M. Lister, I. Barnes, and L. Dalén. 2010. Refugia revisited: individualistic responses of species in space and time. *Proceedings of the Royal Society B: Biological Sciences* 277:661-671.

- Strauss, S. Y., R. E. Irwin, and V. M. Lambrix. 2004. Optimal defence theory and flower petal colour predict variation in the secondary chemistry of wild radish. *Journal of Ecology* 92:132-141.
- Strauss, S. Y., and J. B. Whittall. 2006. Non-pollinator agents of selection on floral traits. Pages 120-138 *In* Harder, L. D., and S. C. H. Barrett, eds. *Ecology and Evolution of Flowers*, Oxford University Press, New York.
- Streisfeld, M. A., and J. R. Kohn. 2005. Contrasting patterns of floral and molecular variation across a cline in *Mimulus aurantiacus*. *Evolution* 59:2548-2559.
- Streisfeld, M. A., and J. R. Kohn. 2007. Environment and pollinator-mediated selection on parapatric floral races of *Mimulus aurantiacus*. *Journal of Evolutionary Biology* 20:122-132.
- Tanaka, Y., N. Sasaki, and A. Ohmiya. 2008. Biosynthesis of plant pigments: anthocyanins, betalains and carotenoids. *The Plant Journal* 54:733-749.
- Thompson, J. N. 1994. *The coevolutionary process*. University of Chicago Press, USA.
- Yoshida, K., Y. Toyama-Kato, K. Kameda, and T. Kondo. 2003. Sepal color variation of *Hydrangea macrophylla* and vacuolar pH measured with a proton-selective microelectrode. *Plant and Cell Physiology* 44:262-268.
- Zhu, C., S. Yamamura, H. Koiwa, M. Nishihara, and G. Sandmann. 2002. cDNA cloning and expression of carotenogenic genes during flower development in *Gentiana lutea*. *Plant Molecular Biology* 48:277-285.
- Zhu, C., S. Yamamura, M. Nishihara, H. Koiwa, and G. Sandmann. 2003. cDNAs for the synthesis of cyclic carotenoids in petals of *Gentiana lutea* and their regulation during flower development. *Biochimica et Biophysica Acta (BBA)-Gene Structure and Expression* 1625:305-308.
- Zhu, C., C. Bai, G. Sanahuja, D. Yuan, G. Farré, S. Naqvi, L. Shi, T. Capell, and P. Christou. 2010. The regulation of carotenoid pigmentation in flowers. *Archives of Biochemistry and Biophysics* 504:132-141.

5.7 APPENDIX

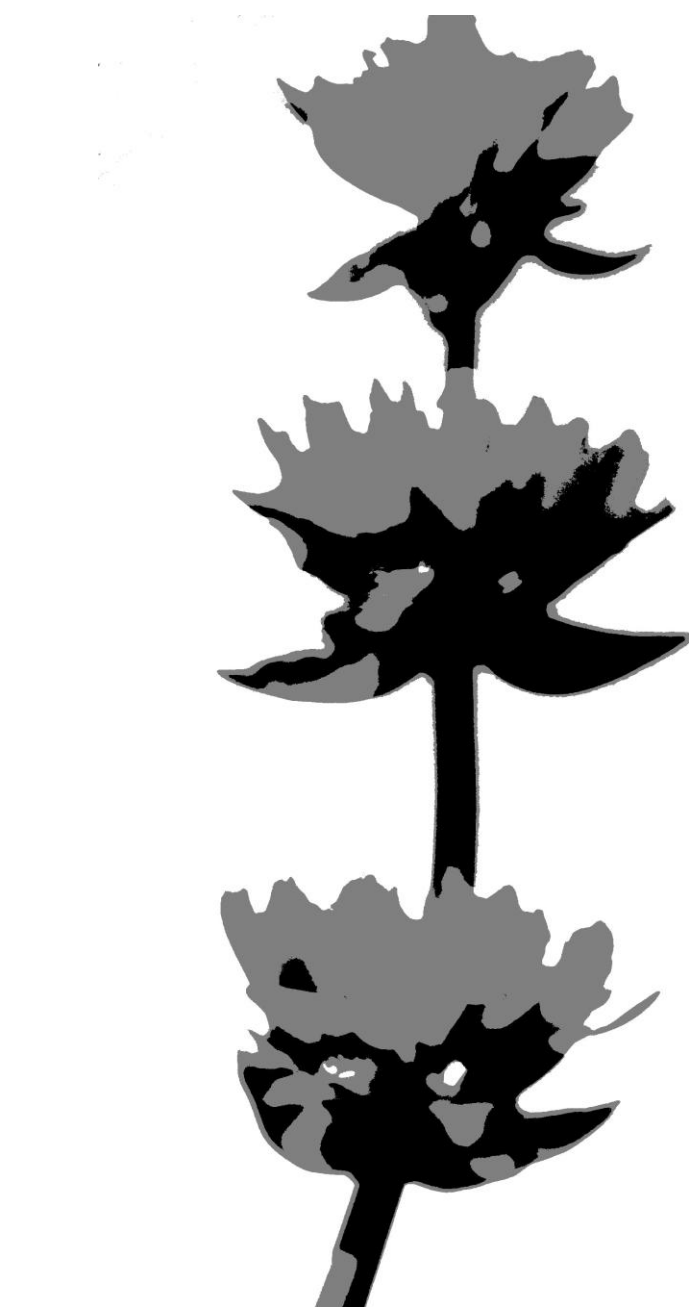
Table A1. Population's traits and coefficients of selection on flower color. We calculated the mean and standard deviation (among parenthesis) for the phenotypic traits and selection coefficients on flower color, in each population. We show the standardized significant ($P < 0.05$) coefficients of selection on flower color. To calculate the standardized selection coefficients, we followed the procedures described in Lande and Arnold (1983). We obtained the total selection differential (S), direct selection gradient (β ; we used as covariates: petal length, petal width, leaf length and the number of flowers); and quadratic (γ_{ii}) and correlational selection (γ_{ij} , γ_{ih} ; i = flower color; j = petal width, h = number of flowers). Note that the quadratic selection coefficients are correctly assessed by doubling the standardized coefficient obtained by the regression (Stinchcombe et al. 2008).

Population	Flower color (PC1)	Leaf length (mm)	Petal length (mm)	Petal width (mm)	Number of flowers	Standardized and significant coefficients of selection on flower color
San Mamede	2.50 (0.37)	238 (41.7)	25.1 (1.50)	6.43 (0.64)	77.3 (21.2)	$S = -0.75$
Queixa	1.24 (0.37)	231 (41.5)	26.1 (2.68)	6.79 (1.24)	74.3 (25.1)	
Loureses	2.37 (0.74)	236 (62.6)	27.0 (2.73)	7.37 (0.71)	77.6 (22.3)	$\gamma_{ii} = 1.95$; $\gamma_{ij} = -1.28$
Cebreiro	2.51 (0.44)	253 (45.6)	24.2 (2.12)	5.80 (1.06)	92.6 (29.0)	$\gamma_{ij} = -0.40$
Ancares	2.29 (0.51)	274 (40.7)	27.2 (2.40)	6.01 (0.80)	89.1 (19.4)	$\beta = -0.21$
Leitariegos	2.41 (0.63)	239 (44.0)	22.7 (2.82)	5.44 (0.87)	76.1 (22.8)	$\gamma_{ii} = -1.13$; $\gamma_{ih} = -1.44$
Torrestío	3.10 (0.74)	244 (47.8)	26.4 (3.11)	5.71 (0.97)	86.6 (26.4)	$\gamma_{ih} = 0.20$
Ventana	3.47 (0.76)	259 (43.3)	26.5 (3.73)	5.20 (1.19)	85.8 (30.0)	$\beta = 0.16$
San Isidro	3.86 (0.57)	257 (52.8)	25.1 (3.23)	4.68 (0.92)	101.5 (41.8)	-
Señales	4.05 (0.45)	243 (37.8)	25.0 (3.21)	4.32 (0.75)	69.3 (40.0)	-
Pontón	3.59 (0.33)	235 (50.2)	25.0 (3.41)	4.57 (0.89)	90.6 (32.3)	-
San Glorio	3.95 (0.39)	223 (70.8)	22.6 (3.23)	4.50 (0.81)	96.5 (38.1)	$\gamma_{ij} = 1.47$
Total	2.95 (0.97)	247 (20.2)	25.2 (3.28)	5.51 (1.21)	85.3 (31.1)	

Table A2. Pollinator assemblage across *Gentiana lutea* populations. We provide the number and percentage (among parenthesis) of visits to *G. lutea* flowers of different morphological groups; some species were joined because differentiation was difficult during census. We joint data of 2010 and 2011; we recorded a total of 7016 visits on 466 plants, during 130 hours of censuses. We included two diversity indexes: S, the species richness, obtained from the number of different groups present in each population; H is the Shannon-Weaver index which considers both, species richness and abundance of species; the higher the values, the higher the diversity.

Population	<i>B. terrestris</i> , <i>B. lucorum</i>	<i>B. hortorum</i> , <i>B. jonellus</i>	<i>B. pratorum</i> , <i>B. soroensis</i>	<i>B. wurflenii</i> , <i>B. lapidarius</i>	<i>B. mesomelas</i>	<i>B. pascuorum</i>	subgen. <i>Psithyrus</i>	<i>Apis</i> spp	S	H
San									6	1.48
Mamede	189 (28)	18 (3)	6 (1)	11 (2)	36 (5)	0	408 (61)	0		
Queixa	304 (35)	5 (1)	52(6)	323 (37)	123(14)	0	70 (8)	3(0)	8	2.03
Loureses	226 (29)	37 (5)	317 (41)	187 (24)	0	7 (1)	4 (1)	0	7	1.85
Cebreiro	182 (49)	27 (7)	35 (9)	17 (5)	5 (1)	0	107 (29)	2 (1)	8	1.94
Ancares	259 (18)	205 (14)	168 (11)	51 (3)	0	15 (1)	768 (52)	0	7	1.92
Leitariegos	199 (51)	57 (15)	50 (13)	10 (3)	63 (16)	2 (1)	5 (1)	5 (1)	8	2.04
Torrestío	112 (78)	8 (6)	3 (2)	0	0	0	0	21 (15)	4	1.04
Ventana	100 (30)	19 (6)	156 (47)	45 (14)	11 (3)	0	0	0	5	1.82
San Isidro	512 (57)	23 (3)	221 (25)	11 (1)	21 (2)	11 (1)	58 (6)	38 (4)	8	1.83
Señales	407 (80)	63 (12)	12 (2)	0	0	10 (2)	14 (3)	2 (0)	4	1.04
Pontón	139 (82)	21 (12)	4 (2)	0	0	0	6 (4)	0	4	0.91
San Glorio	309 (75)	43 (10)	4 (1)	4 (1)	10 (2)	8 (2)	12 (3)	20 (5)	8	1.38
Total	2938 (43)	526 (7)	1028 (15)	659 (9)	269 (4)	53 (1)	1452 (21)	91 (1)	8	





6. DISCUSIÓN



As poboacións de *Gentiana lutea* do noroeste da Península Ibérica difiren en atributos florais e vexetativos, aínda que a variación en caracteres vexetativos ven determinada pola súa correlación cos atributos florais. Particularmente o carácter de maior importancia na variación entre poboacións é a cor das flores. A cor das flores varía tamén dentro de poboacións: tódalas poboacións amosan un rango de variación de cor similar (Fig. 3.2 no capítulo 3, páxina 47). Nalgunhas desas poboacións a variación na reflectancia das flores acontece na zona do espectro que –para o ollo humano– varía de laranxa a amarelo. A variación na cor floral atópase ordenada xeograficamente seguindo un patrón lonxitudinal: no oeste da Cordilleira Cantábrica, as poboacións posúen plantas con flores laranxas; no leste as poboacións posúen plantas con flores amarelas. Máis alá do noso límite de estudo cara o leste, as plantas posúen flores amarelas en toda a súa distribución; Kohlein 1991).

A variación xeográfica nos atributos florais pode acontecer por procesos aleatorios como a deriva xenética, ou por procesos non aleatorios como a selección natural ou a plasticidade fenotípica (Price et al. 2003, Mitchell-Olds et al 2007). A maioría dos trazos florais estudados ata o momento mostran unha alta heredabilidade (Ashman e Majetic 2006) e están suxeitos a selección (Harder e Johnson 2009), incluso aqueles caracteres que mostran plasticidade (por exemplo o tamaño da inflorescencia, Caruso 2006). A pesar de que a deriva xenética pode ser responsable de moita da variación floral atopada na natureza, inda se coñece pouco acerca da importancia deste proceso na evolución floral. En contraste, moitos estudos mostraron que a selección varía xeograficamente e algúns mostran que a selección é a causa da variación floral estruturada xeograficamente (ver referencias en Patiny 2011).

Neste traballo, encontramos que existen presións de selección sobre a cor das flores que veñen determinadas en parte polos polinizadores e os predadores de sementes. Por outra banda, as presións de selección locais sobre a cor das flores varían entre poboacións e ademais, explican parte da estrutura xeográfica da variación na cor das flores –de xeito que nas poboacións de flores laranxas, as plantas máis laranxas son favorecidas, mentres que nas poboacións de flores amarelas, as plantas máis amarelas son beneficiadas. Por tanto, podemos atribuír parte da variación na cor das flores entre poboacións á selección natural.

Tradicionalmente se vincula a variación en características florais á selección exercida polos polinizadores (Fenster 2004, Rausher 2008). Neste traballo atopamos que os polinizadores son responsables de, a lo menos parte, da selección natural sobre a cor das flores. A comunidade de polinizadores varía entre poboacións, de tal xeito que canto máis difiren as poboacións na comunidade de polinizadores, maiores son tamén as diferenzas na cor das flores. Destaca a maior frecuencia de visitas de *B. lapidarius* e polinizadores do subxénero *Psithyrus* en poboacións occidentais. Mais a covariación entre a comunidade e a cor, non implica que sexan os polinizadores os causantes da variación na cor, podería existir un factor común que afectase a ambas, a variación na comunidade de polinizadores e a variación na cor. Sen embargo, sí parece que os polinizadores afectan a variación da cor porque mostran preferencia sobre a cor das flores, preferencia que varía entre poboacións.

A maior parte das especies de polinizadores de *G. lutea* pertencen ó xénero *Bombus* (95%); diferentes especies de abellóns poden variar tanto nas súas preferencias innatas sobre a cor coma no seu sistema visual. Inda que a maioría detectan dificilmente cores vermellas porque carecen de receptores visuais para o vermello (Briscoe e Chittka

2001), hai excepcións coma *B. lapidarius* que posúe sensibilidade para os vermellos (inda que carece de receptores para esta cor; Kugler 1943, Peitsch et al. 1992). Por tanto, as diferenzas na comunidade poderían explicar as diferenzas entre poboacións nas preferencias dos polinizadores sobre a cor das flores.

A evolución das plantas, a partir da cor floral, se estuda maiormente sobre variacións cualitativas (ver Harder e Johnson 2009); mais a evolución das especies pode acontecer sobre variacións cuantitativas máis sutís (Caruso et al. 2010, Renoult et al. 2013). Os polinizadores poden detectar diferenzas cuantitativas na cor das flores que repercuten na produción de sementes (Renoult et al. 2013) e tanto polinizadores coma predadores de sementes poden exercer selección sobre o brillo, antes que sobre a cor (Caruso et al. 2010). Os nosos resultados indican tamén que a selección natural actúa sobre a variación cuantitativa da cor.

Os polinizadores son, probablemente, parcialmente responsables das presións de selección que explican unha parte da variación xeográfica da cor. Máis as presións de selección recollen o efecto de tódolos axentes que afecten a fitness das plantas a través do efecto directo sobre a cor das flores ou indirecto sobre atributos correlacionados coa cor. Tanto outros axentes bióticos, por exemplo herbívoros, coma factores abióticos, poden estar afectando a selección natural sobre a cor das flores.

Neste traballo, alén de estudar o papel dos polinizadores como axentes de selección, consideramos tamén o papel dos predadores de sementes. Encontramos que o seu efecto non permite explicar as diferenzas na cor das flores entre poboacións. Sen embargo, son axentes selectivos sobre a cor das flores a lo menos nunha poboación. Nesta poboación, os predadores de sementes atacan máis ás plantas laranxas, que son as menos polinizadas –ambos axentes favorecen as plantas amarelas, polo que a variación na cor das flores dentro desta poboación non se pode explicar polo efecto combinado de ambos interactuantes. A cor das flores podería estar vinculada á produción de defensas ante o ataque de herbívoros; así, os predadores poderían estar evitando as plantas con certos compostos tóxicos e exercer selección indirecta sobre a cor floral.

A posible relación da cor das flores con defensas químicas, posibilita que a selección estea mediada por outros herbívoros (Irwin et al. 2003, Strauss et al. 2004, Strauss e Whittall 2006). A relación destas plantas con antagonistas non queda reducida ós predadores de sementes: outros herbívoros consumidores de follas, pétalos ou froitos tamén interaccionan con esta especie; algúns deles con efectos directos e intensos sobre o éxito reproductivo ou a supervivencia ó consumir froitos nalgúns poboacións (observacións persoais). Para comprender o papel dos antagonistas como axentes de selección, sería interesante analizar a variación en compostos secundarios e a súa relación coa variación na cor floral. A maioría dos traballos existentes que vinculan o efecto dos herbívoros sobre a variación na cor, versan sobre unha variación na cor relacionada con antocianinas que producen cores rosas-azuis (Irwin et al. 2003, Frey 2004, Caruso et al. 2010, Carlson e Holsinger 2013). Os pigmentos responsables da cor das flores en *G. lutea* son carotenoides (Zhu et al. 2002 e 2003) e, inda que se coñece que esta especie posúe iridoides e flavonoides (Ghedira et al. 2009), compostos vinculados noutras especies á protección ante o ataque de herbívoros (Rauscher 2006, Treutter 2006, Weigend et al. 2006), se descoñece a relación entre os carotenoides e estes compostos secundarios.

Os patróns xeográficos de variación fenotípica poden ser clinais ou en mosaico e poden estar relacionados con factores abióticos (Wolf et al. 2000, Arista et al. 2013), factores bióticos (Thompson 2005) ou ambos (Hampe 2003). Nas plantas polinizadas e

dispersadas por animais, se considera que os factores abióticos afectarán a atributos vexetativos máis que a atributos florais; sen embargo, os factores bióticos poden afectar tanto os caracteres reprodutivos (é dicir, as flores: Boyd 2002; os froitos: Hampe 2003, Sobral et al 2010, pero ver Traveset et al. 2004) coma os vexetativos (Berenbaum e Zangerl 1998, Snyder e Linhart 1998).

Sendo que a cor das flores varía segundo un patrón lonxitudinal, aqueles factores abióticos que varíen lonxitudinalmente son candidatos para ser forzas de selección sobre a cor das flores; por exemplo a precipitación ou a temperatura (Rodríguez-Puebla et al. 1998, AEMET IM 2011), que varían de tal forma que a sequía estival incrementa cara occidente na Cordilleira Cantábrica (Rivas-Martínez et al. 1984). Ademais estes factores poderían repercutir na variación da cor das flores, por medio de influenciar na comunidade de interactuantes (Steffan-Dewenter et al. 2002, Bale et al. 2002, Ploquin et al. 2013). Así mesmo o solo parece diferenciarse de leste a oeste: o occidente posúe substratos ácidos e o oriente posúe substratos básicos (Moreno-Saiz et al. 2013). Características do solo, coma poden se-lo pH ou a concentración de aluminio, poden provocar variación na cor das flores (Ma et al. 2001, Yoshida et al. 2003). Sen embargo, encontramos que as diferenzas fenotípicas entre poboacións tanto en atributos florais coma nos vexetativos non se correlacionan co pH do solo nin coa temperatura, radiación ou precipitacións medias estivais.

A selección natural, exercida presumiblemente polos polinizadores, inflúe en parte na variación da cor das flores de *Gentiana lutea* entre poboacións, xunto con outros factores, como aqueles relacionados coa localización xeográfica. O fluxo xénico entre poboacións próximas podería explicar a variabilidade na cor das flores. Sen embargo, non parece que o fluxo xénico sexa moi relevante nas poboacións estudadas: a entrada de pole dende poboacións próximas debe ser baixa porque as poboacións se atopan rodeadas por cumios de montañas que as separan doutras poboacións. Mencionar tamén que a estrutura xeográfica na variación da cor podería estar marcada por procesos históricos. Os movementos migratorios en especies de montaña marcan a distribución e illamento das poboacións (Stewart et al. 2010, Alarcon et al. 2012). As especies de alta montaña do norte da Península Ibérica, durante os períodos cálidos do Cuaternario, soben en altitude ficando illadas; durante os períodos fríos baixan ós vales onde entran en contacto. Estes movementos migratorios propician o illamento xenético e a diversificación (Stewart et al. 2010, Alarcon et al. 2012). Sen embargo, a variabilidade nos atributos florais (particularmente na cor) é moito maior que a variabilidade nos caracteres vexetativos e, ó controlar pola variación floral, non atopamos estrutura xeográfica na variación vexetativa. Estes feitos fannos pensar que os procesos aleatorios –debido a procesos históricos ou deriva xenética– non causaron esta variación que parece provocada por axentes de selección sobre os trazos florais. Se estes procesos aleatorios xogasen un rol determinante, esperaríamos que a variación floral e vexetativa seguise patróns similares. Por suposto, estes procesos e a selección natural ou a plasticidade fenotípica non son mutuamente excluíntes e o máis probable é que todos eles xoguen ou teñan xogado un rol na variación fenotípica de *G. lutea*.

Para rematar queremos remarcar que este estudo é de natureza observacional, e que por tanto, non se poden resolver as relacións de causalidade; os patróns observados son de tipo correlativo. Para abarcar as relacións causa-efecto, serían axeitados experimentos coma transplantes recíprocos (Kawecki e Ebert 2004) ou manipulacións artificiais de caracteres (Campbell 2009). Consideramos que os estudos observacionais en condicións naturais son imprescindibles para comprender a natureza. Sen estes estudos observacionais –a escalas espaciais ecoloxicamente relevantes– correríamolo

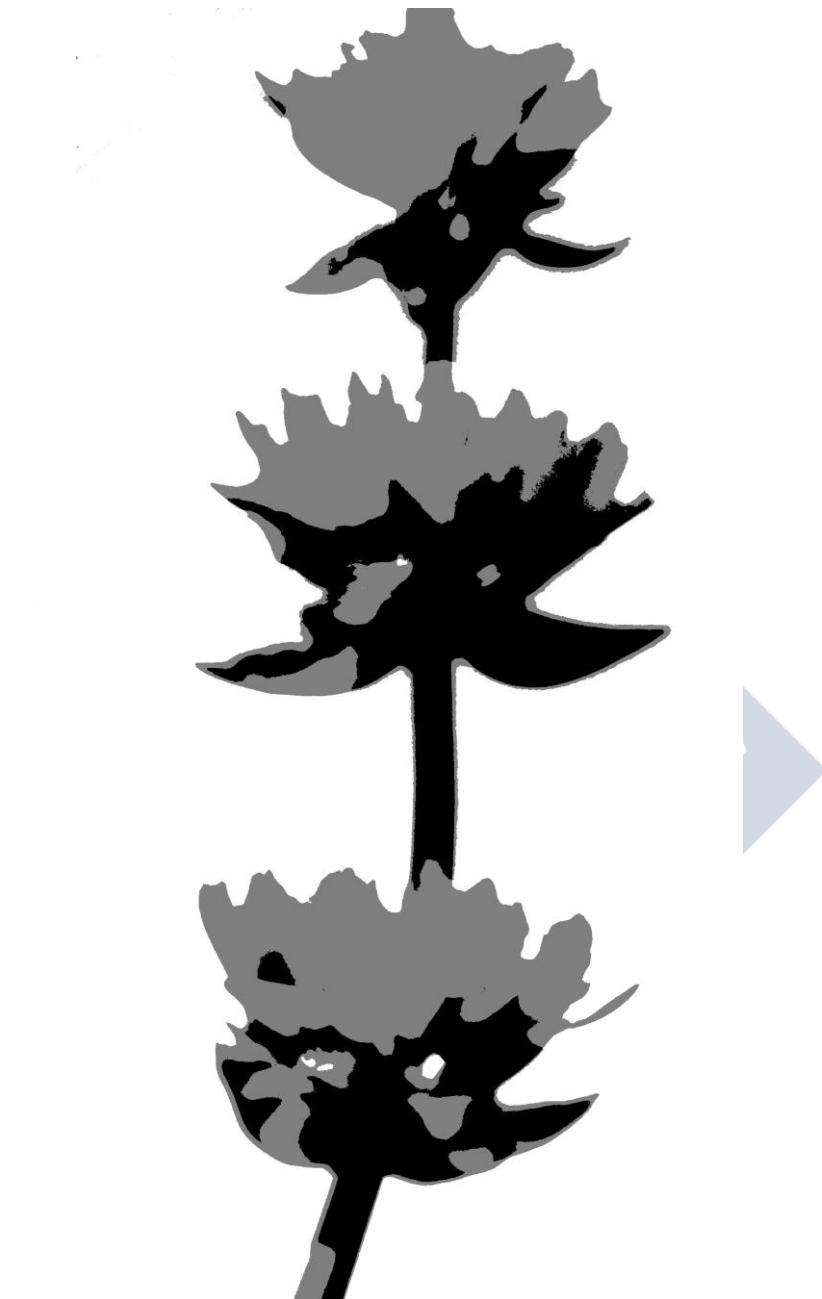
risco de interpretar a natureza partindo, exclusivamente, de coñecementos adquiridos a partir de especies modelo e sistemas experimentais. Estudar a selección natural sobre a cor das flores en *Gentiana lutea* e mostrar que polinizadores e predadores poden ser axentes de selección, afectando as diferenzas na cor entre poboacións, aumenta o noso coñecemento sobre a natureza adaptativa da cor floral e en xeral sobre a selección natural exercida por animais mutualistas e antagonistas sobre as características das plantas.

6.1 REFERENCIAS

- AEMET-IM. 2011. Iberian Climate Atlas–Air temperature and precipitation (1971-2000). Agencia Estatal de Meteorología, Ministerio de Medio Ambiente y Medio Rural y Marino e Instituto de Meteorologia de Portugal (eds). ISBN: 978-84-7837-079-5.
- Alarcón, M., P. Vargas, L. Sáez, J. Molero, e J. J. Aldasoro. 2012. Genetic diversity of mountain plants: Two migration episodes of Mediterranean *Erodium* (Geraniaceae). *Molecular Phylogenetics and Evolution* 63:866-876.
- Arista, M., M. Talavera, R. Berjano, and P. L. Ortiz. 2013. Abiotic factors may explain the geographical distribution of flower colour morphs and the maintenance of colour polymorphism in the scarlet pimpernel. *Journal of Ecology* 101:1613-1622.
- Ashman, T., and C. Majetic. 2006. Genetic constraints on floral evolution: a review and evaluation of patterns. *Heredity* 96:343-352.
- Bale, J. S., G. J. Masters, I. D. Hodkinson, C. Awmack, T. M. Bezemer, V. K. Brown, J. Butterfield, A. Buse, J. C. Coulson, e J. Farrar. 2002. Herbivory in global climate change research: direct effects of rising temperature on insect herbivores. *Global Change Biology* 8:1-16.
- Berenbaum, M. R., e A. R. Zangerl. 1998. Chemical phenotype matching between a plant and its insect herbivore. *Proceedings of the National Academy of Sciences of the United States of America* 95:13743-13748.
- Boyd, A. 2002. Morphological analysis of Sky Island populations of *Macromeria viridiflora* (Boraginaceae). *Systematic Botany* 27:116-126.
- Briscoe, A. D., e L. Chittka. 2001. The evolution of color vision in insects. *Annual Review of Entomology* 46:471-510.
- Campbell, D. R. 2009. Using phenotypic manipulations to study multivariate selection of floral trait associations. *Annals of Botany* 103:1557-1566.
- Carlson, J. E., e K. E. Holsinger. 2013. Direct and indirect selection on floral pigmentation by pollinators and seed predators in a color polymorphic South African shrub. *Oecologia* 171:905-919.
- Caruso, C. M. 2006. Plasticity of inflorescence traits in *Lobelia siphilitica* (Lobeliaceae) in response to soil water availability. *American Journal of Botany* 93:531-538.
- Caruso, C. M., S. L. Scott, J. C. Wray, e C. A. Walsh. 2010. Pollinators, herbivores, and the maintenance of flower color variation: a case study with *Lobelia siphilitica*. *International Journal of Plant Science* 171:1020-1028.
- Fenster, C. B., W. S. Armbruster, P. Wilson, M. R. Dudash, e D. James. 2004. Pollination syndromes e floral specialization. *Annual Review of Ecology, Evolution and Systematics* 35:375-403.
- Frey, F. M. 2004. Opposing natural selection from herbivores and pathogens may maintain floral-color variation in *Claytonia virginica* (Portulacaceae). *Evolution* 58:2426-2437.
- Ghedira, K., P. Goetz, R. Le Jeune, e C. Faivre. 2009. *Gentiana lutea* L. (Gentianaceae). *Phytothérapie* 7:172-176.

- Hampe, A. 2003. Large-scale geographical trends in fruit traits of vertebrate-dispersed temperate plants. *Journal of Biogeography* 30:487-496.
- Harder, L. D., e S. D. Johnson. 2009. Darwin's beautiful contrivances: evolutionary and functional evidence for floral adaptation. *New Phytologist* 183:530-545.
- Irwin, R. E., S. Y. Strauss, S. Storz, A. Emerson, e G. Guibert. 2003. The role of herbivores in the maintenance of a flower color polymorphism in wild radish. *Ecology* 84:1733-1743.
- Kawecki, T. J., e D. Ebert. 2004. Conceptual issues in local adaptation. *Ecology Letters* 7:1225-1241.
- Kohlein, F. 1991. *Gentians*. Timber Press Inc, Portland, Oregon, USA.
- Kugler, H. 1943. Hummeln als Blütenbesucher. *Ergebnisse der Biologie* 19:323.
- Lande, R., e S. J. Arnold. 1983. The measurement of selection on correlated characters. *Evolution* 37:1210-1226.
- Ma, J. F., P. R. Ryan, e E. Delhaize. 2001. Aluminium tolerance in plants and the complexing role of organic acids. *Trends in Plant Science* 6:273-278.
- Mitchell-Olds, T., J. H. Willis, e D. B. Goldstein. 2007. Which evolutionary processes influence natural genetic variation for phenotypic traits? *Nature Reviews Genetics* 8:845-856.
- Moreno-Saiz, J. C., M. Donato, L. Katinas, J. V. Crisci, and P. Posadas. 2013. New insights into the biogeography of south-western Europe: spatial patterns from vascular plants using cluster analysis and parsimony. *Journal of Biogeography* 40:90-104.
- Patiny, S. 2011. *Evolution of plant-pollinator relationships*. Cambridge University Press, United Kingdom.
- Peitsch, D., A. Fietz, H. Hertel, J. de Souza, D. F. Ventura, e R. Menzel. 1992. The spectral input systems of hymenopteran insects and their receptor-based colour vision. *Journal of Comparative Physiology A* 170:23-40.
- Ploquin, E. F., J. M. Herrera, e J. R. Obeso. 2013. Bumblebee community homogenization after uphill shifts in montane areas of northern Spain. *Oecologia* 173:1649-1660.
- Price, T. D., A. Qvarnstrom, e D. E. Irwin. 2003. The role of phenotypic plasticity in driving genetic evolution. *Proceedings Biological Sciences / The Royal Society* 270:1433-1440.
- Rausher, M. D. 2006. The evolution of flavonoids and their genes. Pages 175-211 *En* Grotewold, E., ed. *The science of flavonoids*, Springer, Ohio, USA.
- Rausher, M. D. 2008. Evolutionary transitions in floral color. *International Journal of Plant Science* 169:7-21.
- Renoult, J., M. Thomann, H. Schaefer, e P. Cheptou. 2013. Selection on quantitative colour variation in *Centaurea cyanus*: the role of the pollinator's visual system. *Journal of Evolutionary Biology* 26:2415-2427.
- Rivas-Martínez, S., T. E. Díaz, J. A. F. Prieto, J. Loidi e A. Penas. 1984. *La Vegetación de la Alta Montaña Cantábrica: Los Picos de Europa*. Ediciones Leonesas. León.
- Rodríguez-Puebla, C., A. Encinas, S. Nieto, e J. Garmendia. 1998. Spatial and temporal patterns of annual precipitation variability over the Iberian Peninsula. *International Journal of Climatology* 18:299-316.
- Snyder, M., e Y. B. Linhart. 1998. Subspecific selectivity by a mammalian herbivore: geographic differentiation of interactions between two taxa of *Sciurus aberti* and *Pinus ponderosa*. *Evolutionary Ecology* 12:755-765.
- Sobral, M., A. R. Larrinaga, e J. Guitián. 2010. Do seed-dispersing birds exert selection on optimal plant trait combinations? Correlated phenotypic selection on the fruit

- and seed size of hawthorn (*Crataegus monogyna*). *Evolutionary Ecology* 24:1277-1290.
- Steffan-Dewenter, I., U. Münzenberg, C. Bürger, C. Thies, e T. Tschamntke. 2002. Scale-dependent effects of landscape context on three pollinator guilds. *Ecology* 83:1421-1432.
- Stewart, J. R., A. M. Lister, I. Barnes, e L. Dalén. 2010. Refugia revisited: individualistic responses of species in space and time. *Proceedings of the Royal Society B: Biological Sciences* 277:661-671.
- Strauss, S. Y., R. E. Irwin, e V. M. Lambrix. 2004. Optimal defence theory and flower petal colour predict variation in the secondary chemistry of wild radish. *Journal of Ecology* 92:132-141.
- Strauss, S. Y., e J. B. Whittall. 2006. Non-pollinator agents of selection on floral traits. Pages 120-138 *En Harder, L. D., e S. C. H. Barret, eds. Ecology and Evolution of Flowers*, Oxford University Press, New York.
- Thompson, J. N. 2005. The geographic mosaic of coevolution. University of Chicago Press, Chicago, USA.
- Traveset, A., M. F. Willson, e M. Verdú. 2004. Characteristics of fleshy fruits in southeast Alaska: phylogenetic comparison with fruits from Illinois. *Ecography* 27:41-48.
- Treutter, D. 2006. Significance of flavonoids in plant resistance: a review. *Environmental Chemistry Letters* 4:147-157.
- Weigend, M., J. Kufer, e A. A. Muller. 2000. Phytochemistry and the systematics and ecology of Loasaceae and Gronoviaceae (Loasales). *American Journal of Botany* 87:1202-1210.
- Wolf, A. T., S. P. Harrison, e J. L. Hamrick. 2000. Influence of habitat patchiness on genetic diversity and spatial structure of a serpentine endemic plant. *Conservation Biology* 14:454-463.
- Yoshida, K., Y. Toyama-Kato, K. Kameda, e T. Kondo. 2003. Sepal color variation of *Hydrangea macrophylla* and vacuolar pH measured with a proton-selective microelectrode. *Plant and Cell Physiology* 44:262-268.
- Zhu, C., S. Yamamura, H. Koiwa, M. Nishihara, e G. Sandmann. 2002. cDNA cloning and expression of carotenogenic genes during flower development in *Gentiana lutea*. *Plant Molecular Biology* 48:277-285.
- Zhu, C., S. Yamamura, M. Nishihara, H. Koiwa, e G. Sandmann. 2003. cDNAs for the synthesis of cyclic carotenoids in petals of *Gentiana lutea* and their regulation during flower development. *Biochimica et Biophysica Acta (BBA)-Gene Structure and Expression* 1625:305-308.



7.CONCLUSIONES



CONCLUSIONS

1. Populations of *Gentiana lutea* at the west of the Cantabrian Range (southwest Europe), differ mainly in flower color. Despite there is also variation on vegetative traits, this variation occurs due to a correlation among vegetative and floral traits.

2. Variation on flower color occurs within and among populations of *G. lutea*. Within-population variation has a similar magnitude across populations –differing on the mean values of floral color. Among population variation follows a longitudinal pattern: yellow-flowered plants grow in eastern populations and orange-flowered plants grow in western populations.

3. Among population differences in climatic and edaphic abiotic factors (soil pH, temperature, radiation and rainfall) are not correlated to phenotypic differences among populations.

4. Pollinators and seed predators exert selection on color within a population. The combined effect of both agents does not explain the color variability because both agents favor the yellow-flowered plants.

5. Pollinator preferences on color vary among populations and differences in the community of pollinators are related to the differences on color among populations.

6. Natural selection, exerted in part by pollinators, varies among populations and influences the geographic structure of color variation. Flower color in *G. lutea* is locally adapted to selective pressures.

CONCLUSIÓN

1. As poboacións de *Gentiana lutea* do oeste da Cordilleira Cantábrica (suroeste de Europa), difiren sobre todo na cor floral. A pesar de que tamén existe variación en atributos vexetativos, esta variación acontece debido á correlación entre caracteres vexetativos e florais.

2. A variación na cor floral acontece dentro e entre poboacións de *G. lutea*. A variación dentro das poboacións posúe unha magnitude similar que entre poboacións –diferindo nos valores medios da cor floral. Entre poboacións, a variación segue un patrón lonxitudinal: nas poboacións orientais medran plantas con flores amarelas, nas poboacións occidentais as plantas posúen flores laranxas.

3. As diferenzas entre poboacións nos factores abióticos climáticos e edáficos (pH do solo, temperatura, radiación e precipitación) non están correlacionadas coas diferenzas fenotípicas entre poboacións.

4. Polinizadores e predadores de sementes exercen selección sobre a cor nunha poboación. O efecto combinado de ambos axentes non permite explicar a variabilidade da cor nesta poboación xa que ambos axentes favorecen as plantas de flores amarelas.

5. As preferencias dos polinizadores sobre a cor das flores varían entre poboacións e as diferenzas na comunidade de polinizadores están relacionadas coas diferenzas na cor das flores entre poboacións.

5. A selección natural, ejercida en parte por polinizadores, varía entre poboacións e inflúe na estrutura xeográfica da variación da cor en *Gentiana lutea*. A cor das flores en *G. lutea* está localmente adaptada ás presións de selección.

CONCLUSIONES

1. Las poblaciones de *Gentiana lutea* del oeste de la Cordillera Cantábrica (suroeste de Europa), difieren sobretudo en el color de las flores. A pesar de que también existe variación geográfica en rasgos vegetativos, esta variación ocurre debido a la correlación entre caracteres vegetativos y florales.

2. La variación en el color floral ocurre dentro y entre poblaciones de *G. lutea*. La variación dentro de poblaciones posee una magnitud similar que entre poblaciones – difiriendo en el valor medio del color. Entre poblaciones, la variación sigue un patrón longitudinal: en las poblaciones orientales crecen plantas con flores amarillas, en las poblaciones occidentales las plantas son de floración naranja.

3. Las diferencias entre poblaciones en los factores abióticos climáticos y edáficos (pH del suelo, temperatura, radiación y precipitación) no se encuentran correlacionadas con las diferencias fenotípicas entre poblaciones.

4. Polinizadores y predadores de semillas ejercen selección sobre el color en una población. El efecto combinado de ambos agentes no permite explicar la variabilidad del color en esta población ya que ambos agentes favorecen las plantas de flores amarillas.

5. Las preferencias de los polinizadores sobre el color de las flores varían entre poblaciones y las diferencias en la comunidad de polinizadores están relacionadas con las diferencias en el color de las flores entre poblaciones.

6. La selección natural, ejercida en parte por polinizadores, varía entre poblaciones e influye en la estructura geográfica de la variación del color en *Gentiana lutea*. El color de las flores en *G. lutea* está localmente adaptado a las presiones de selección.



As poboacións de *Gentiana lutea* do oeste da Cordilleira Cantábrica difiren en atributos florais e vexetativos, sobre todo na cor das flores. A cor das flores desta especie varía de laranxa a amarelo, de oeste a leste. Inda que a cor inflúe nas preferencias dos polinizadores e predadores de sementes; a selección natural, exercida probablemente polos polinizadores e non polos predadores, explica parte da variación na cor das flores entre as poboacións de *Gentiana lutea*.

