

# Immunocompetence, developmental stability and wingspot size in the damselfly *Calopteryx splendens* L.

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*Calopteryx splendens* males exhibit a remarkable variation in wing pigmentation both within and between populations. In this study, we examined whether the wingspots of male *C. splendens* are related to male quality. We measured the nylon implant encapsulation rate for 85 males and found that males with larger wingspots had a faster encapsulation rate, indicating a better immunocompetence. We also found that the encapsulation rate was positively correlated with the density of haemocytes in the haemolymph. Another measurement of male quality, fluctuating asymmetry of wingspots, correlated negatively with the size of the wingspots. Males with asymmetrical wingspots also had lower encapsulation rates than more symmetrical males. Our results suggest that the size of wingspot is an indicator of male quality in *C. splendens*.

**Keywords:** asymmetry; *Calopteryx splendens*; damselfly; encapsulation rate; fluctuating asymmetry; immunocompetence

## 1. INTRODUCTION

Various ornaments, such as badges, have been observed to influence the outcome of contests for resources in many animal species (reviewed in Andersson 1994). These ornaments may be correlated with the phenotypic condition and fighting ability of the possessor, permitting strongly ornamented individuals to win contests through display without escalated fights (Andersson 1982; Maynard Smith & Harper 1988). To signal fighting ability honestly over evolutionary time, the cost of a given size of the ornament must be higher for lower-quality males (Nur & Hasson 1984; Andersson 1986; Zeh & Zeh 1988; Grafen 1990; Johnstone 1995; Kotiaho 2000; but see Getty 1998). A general prediction is that under these conditions, higher-quality males should develop larger or brighter ornaments (Zeh & Zeh 1988; Grafen 1990).

Several studies on birds suggest that immune function may play an important role in maintaining honesty of secondary sexual characters (Gustafsson *et al.* 1994; Saino & Møller 1996). In contrast to vertebrates, insects have only innate immunity, involving a humoral and cellular response. In the cellular response, haemocytes attach to parasites (Gupta 1986). In the process, the foreign object may become completely encapsulated in layers of haemocytes, which die and subsequently become melanized, thereby isolating the intruder from host tissue (Ratcliffe 1993). The humoral system, on the other hand, is comprised of a myriad of soluble proteins and enzyme cascades, which play important roles in recognizing, signalling and attacking foreign targets (Leonard *et al.* 1985), and probably in coordinating the cellular responses (Pech & Strand 1995). The simplest, and probably the most informative, way to assay insect immune response is to measure the magnitude of the cellular encapsulation response to a novel and standardized 'pathogen' like a

nylon monofilament (Köning & Schmid-Hempel 1995; Schmid-Hempel & Schmid-Hempel 1998; Siva-Jothy *et al.* 1998).

Fluctuating asymmetry (FA) has been used as a measure of individual quality (Møller 1997; Møller & Swaddle 1997; but see Palmer 1999; Simmons *et al.* 1999; Thornhill *et al.* 1999). FA is a measure of stochastic differences between the right and left halves of bilaterally symmetrical organisms (Palmer & Strobeck 1986). It represents a measure of developmental instability, which in turn is an indicator of how good the genome is at controlling the processes of normal development. Thus, the ability to produce symmetrical ornaments is likely to be an indicator of male quality (Møller & Swaddle 1997). Many studies have shown that there is a negative relationship between the size of a sexually selected trait and the degree of FA of the trait, because male ability to produce a large ornament appears to be related to its ability to produce symmetrical ornaments, both traits being indicators of organism quality (reviewed in Møller & Swaddle 1997).

Males of the banded agrion, *Calopteryx splendens*, have large, iridescent, blue wingspots, which fit well the description of a classic secondary sexual character (cf. Darwin 1871), and although males exhibit remarkable geographical variation in wing coloration, there is huge variation among males even within a single population (Valle 1937, 1952; Dumont *et al.* 1993). Previous observational and experimental studies with a closely related species, *Hetaerina americana* (Calopterygidae), have revealed that variation in male wing coloration is maintained by competition among males for mating territories, not by female choice (Grether 1996a,b, 1997). Grether (1996a,b) found that males with naturally or artificially large wingspots were more successful at holding territories and consequently mated at higher rates than males with relatively small wingspots. He found also that males with enlarged spots had higher mortality rates than both unmanipulated and sham-manipulated controls. He

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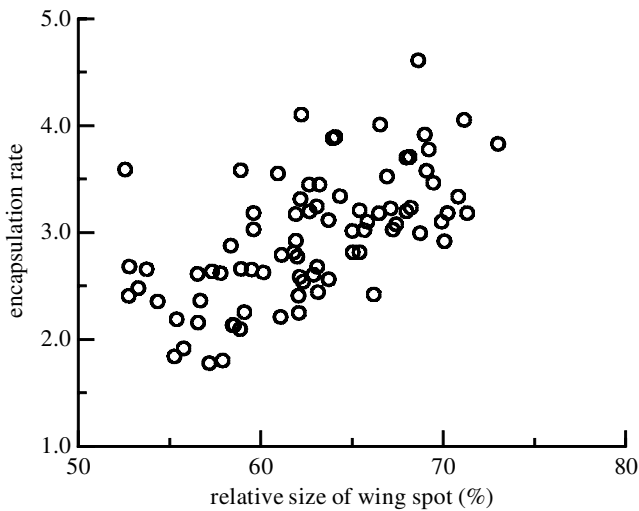


Figure 1. Relationship between the relative size of wingspot and encapsulation rate in *C. splendens* males.

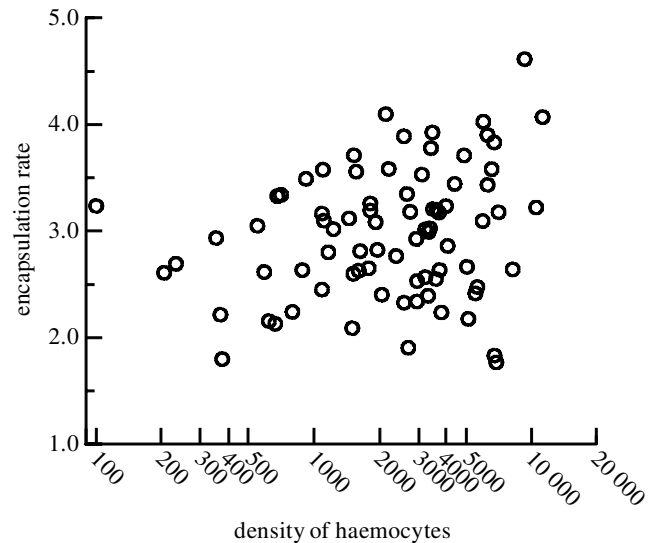


Figure 2. Relationship between the density of haemocytes and encapsulation rate in *C. splendens*. Density of haemocytes is  $\log_{10}$  transformed.

concluded therefore that those ornaments could have evolved as honest indicators of fighting ability, because they provided costs that make deceptive signalling unprofitable (Grether 1997).

The aim of our study was to test whether the male wing coloration of *C. splendens* is an indicator of male quality. As a measure of male quality we used males' immunocompetence and FA. We predicted that males with larger wingspots would have a faster encapsulation rate and more symmetrical wingspots.

## 2. METHODS

We collected 85 males of *C. splendens* between 2 and 6 July 1999 from Creek Neulajoki near the city of Jyväskylä (62°16' N, 25°30' W) in southern Finland. The damselflies were captured with a butterfly net and were kept in individual black plastic containers in a portable electrical cooling box ( $12 \pm 2^\circ\text{C}$ ). Because we did not commence our observations at the beginning of the flying season it was not possible to determine absolute age of males by the date of marking. Males were therefore aged by assigning them to one of the age categories defined by the stiffness of the leading edge of the wings, which increases with age (see Siva-Jothy & Tsubaki 1994; Plaistow & Siva-Jothy 1996). In the present study, we collected only males that belonged to age class 2. In the laboratory, we measured body mass with an accuracy of 0.1 mg.

To challenge the damselfly immune system, we inserted a 1.7–1.8 mm long piece of nylon monofilament (diameter: 0.1 mm) through the fourth abdominal pleura on the dorsal side of the sternal–tergal margin. The males' immune system was allowed to react to this object for 5 h (this time was chosen because our preliminary study showed that it produces the largest differences between individuals), while the insects were kept in individual black film roll cans at constant room temperature ( $22 \pm 1^\circ\text{C}$ ). The implant was then removed and dried. Three implants could not be recovered. The removed monofilaments were photographed with a microscope from three different directions. All photographs of the implants were taken in standardized set-up with 'control implants' (an unused piece of nylon to correct for differences in film development) and

analysed with a densitometer. We divided implants into four sections and to determine the degree of encapsulation rate of each implant, the optical densities of red (R, filter 700 nm), green (G, filter 546.1 nm) and blue (B, filter 435.8 nm) were measured at those four sections. Total colour density (R + G + B) was calculated for each section and the appropriate measures for implants were calculated from the mean value of all three different photographs. To measure repeatability we took another set of photographs from 16 randomly chosen implants and independently analysed them with a densitometer. The repeatability ( $R$ ) of this method was high ( $R = 0.98$ ,  $F_{17,18} = 105.11$ ,  $p < 0.001$ ) (see Krebs 1989). To measure haemocyte density, we counted the relative numbers of circulating haemocytes in the haemolymph. Before removing the implant, damselflies were punctured at the fifth abdominal pleura on the dorsal side of the sternal–tergal margin with a sterile needle to obtain haemolymph, and the haemolymph was collected from the puncture into a glass micropipette containing heparin. Haemolymph was smeared on a slide, air-dried and fixed using methanol. The slides were stained using Diff Quick<sup>1</sup> (Dade Diagnostika GmbH, München, Germany) and then counted using the microscope ( $\times 1000$  magnification). All haemocyte types encountered were pooled to total haemocyte counts. We used  $\log_{10}$  transformation for the haemocyte density, after which the data satisfied the assumptions of the statistical tests.

To measure parasite burden, we assessed the number of eugregarine trophozoites in a male's mid-gut by removing the entire gut carefully and making light microscope thin-section preparations (see Siva-Jothy & Plaistow 1999). The wings were cut off and placed between two microscope slides taped together. The wings were scanned and printed to paper five times larger than normal size. The total area of the wingspots was measured by weighing the cut-out mass of the paper shape. The areas of unpigmented spots in wings were measured in a similar way. The FA of wingspot size was estimated by calculating the absolute differences between the left and right sides of each character. Asymmetry measurements were not taken from four individuals with damaged wings. The properties of FA measurements were assessed following the procedure of Swaddle *et al.*

Table 1. Correlation coefficients ( $r$ ) of encapsulation rate and density of haemocytes ( $\log_{10}$  transformed) in relation to body mass, length of hind wing, the size of wingspot, unpigmented area of wing spot and fluctuating asymmetry(The number of individuals is denoted by  $n$ , and the significance of correlation coefficient by  $p$ .)

	encapsulation rate			density of haemocytes		
	$r$	$n$	$p$	$r$	$n$	$p$
body mass	-0.13	82	0.24	-0.09	83	0.42
wing length	-0.04	82	0.70	-0.04	83	0.72
size of wingspot	-0.62	82	< 0.001	0.02	81	0.88
unpigmented area	-0.22	82	0.047	-0.08	83	0.46
ornament asymmetry	-0.23	78	0.040	0.05	79	0.63

(1994). There was no evidence of directional asymmetry because the frequency distribution of right minus left values did not differ from a normal distribution with a mean of zero. Repeatability of measurements of wingspot asymmetry was high ( $R = 0.77$ ,  $F_{15,16} = 7.70$ ,  $p < 0.001$ ) (see Krebs 1989).

### 3. RESULTS

The area of the wingspots varied from 53 to 73% of the wing area. Similarly, the encapsulation rate varied considerably between males (figures 1 and 2). There was a strong positive relationship between relative size of wingspot and encapsulation rate ( $r = 0.62$ ,  $n = 82$ ,  $p < 0.001$ ; figure 1). The density of haemocytes in males was on average  $3100 \mu\text{l}^{-1}$  (s.d. = 2400, range 100–11 000) and also positively correlated with encapsulation rate ( $r = 0.25$ ,  $n = 80$ ,  $p = 0.027$ ; figure 2). However, there was no correlation between the relative size of wingspot and the density of haemocytes ( $r = -0.03$ ,  $n = 83$ ,  $p = 0.82$ ). There was a negative correlation between the ornament size and ornament asymmetry ( $r = -0.25$ ,  $n = 81$ ,  $p = 0.035$ ). Furthermore, the asymmetry of wingspots was negatively correlated with encapsulation rate ( $r = -0.23$ ,  $n = 78$ ,  $p = 0.040$ ).

The proportion of unpigmented area in wingspots varied from 0 to 4% and males with more unpigmented areas in wingspots had a lower encapsulation rate ( $r = 0.22$ ,  $n = 82$ ,  $p = 0.047$ ). Other morphological measurements, length of hind wings and body mass, did not correlate with encapsulation rate, nor with the density of haemocytes (table 1).

We found neither ectoparasites nor eugregarines from males of *C. splendens*.

### 4. DISCUSSION

The main result of the present study was the positive relationship between the size of wingspot and the encapsulation rate in male *C. splendens*, indicating that more-ornamented males have a better immunocompetence. Previous studies with another damselfly have revealed that male wing coloration pigments are constituted from melanin (Hooper *et al.* 1999). Melanin is a key component of the humoral immune system and a central part of the encapsulation response (Ratcliffe 1993). So there might be a trade-off between ornamentation and immunocompetence. Thus only males in good condition are able to produce large ornaments (see Folstad & Karter 1992).

It is possible that the size of wingspots is correlated with immunocompetence because only males of high phenotypic quality are able to cope with the costs of producing efficient immune responses. On the other hand, males with larger wingspots might pay some other costs, if the ornament size reliably reflects male quality. One of those costs might be increased conspicuousness to visually orientating predators. An increased risk of predation on visually signalling males has been demonstrated in many animals (see Andersson 1994; Zuk & Kolluru 1998). It is possible that wingspots may increase conspicuousness also to visually orientating prey (Grether & Grey 1996), thereby limiting male feeding opportunities. Furthermore, maintaining large ornaments may be costly due to increased aggression by other males (Rohwer & Rohwer 1978).

It is not known whether wingspots are maintained by female choice, male–male competition or both. However, a previous work suggested that female *C. splendens xanthostoma* prefer to mate with males that have large wingspots (Siva-Jothy 1999). Thus, in favouring males that produce large ornaments, females may select males with a greater ability to encapsulate pathogens (see Ryder & Siva-Jothy 2000).

Surprisingly, we did not find any macroparasites from *C. splendens* in this study, not even eugregarines, which are cosmopolitan mid-gut parasites of damselflies (Åbro 1996). The infection with eugregarine trophozoites during the teneral life-history stage is known to affect the ability of damselflies to accumulate fat, and consequently reduce their ability to fight for, and maintain, a territory when they become reproductively active (Siva-Jothy & Plaistow 1999). The absence of parasites in our study population may have reduced confounding factors, but it may have also affected the trade-off between ornamentation and immunocompetence.

We found a weak positive relationship between encapsulation rate and the density of haemocytes, which has not been found in previous studies in insects. Cellular encapsulation responses are possibly initiated by random contact of the circulating haemocytes with foreign objects (Millar & Ratcliffe 1994). Thus it was not surprising to find that encapsulation rate was correlated with the density of haemocytes. However, the correlation between the density of haemocytes and the size of wingspots was not significant in the present study.

As in many previous studies (reviewed in Møller & Swaddle 1997), we found a negative relationship between

the size of ornaments and degree of FA, indicating that the size of wingspots might be an indicator of a male's quality. We also found that males with more symmetrical wingspots had better encapsulation rates. Similarly, Lagesen & Folstad (1998) found that male reindeer with more symmetrical antlers had better immunocompetence. However, previous studies on birds have not found any correlation between immunocompetence and FA (Møller *et al.* 1996; Kimball *et al.* 1997). Increased FA is thought to be a result of many environmental and genetic stressors during ontogeny (Møller & Swaddle 1997). Thus, our results indicate that those stressors may also influence the immunocompetence. Our results support the idea that FA may be used as a measure of individual quality. Furthermore, males with unpigmented spots on their wings showed reduced encapsulation rates in the present study, which suggests that developmental instability may be associated with decreased immunocompetence.

In conclusion, our study has shown that the size and symmetry of male wingspots in *C. splendens* may work as an indicator of individual quality. Our results support the hypothesis that high-quality males will develop both large ornamental traits and relatively symmetrical bilateral traits. In addition, our finding that males with more symmetrical wingspots had higher encapsulation rates indicates that exposure to developmental disturbances may be associated with decreased immunocompetence.

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## REFERENCES

- Åbro, A. 1996 Gregarine infection of adult *Calopteryx virgo* L. (Odonata: Zygoptera). *J. Nat. Hist.* **30**, 855–859.
- Andersson, M. 1982 Sexual selection, natural selection and quality advertisement. *Biol. J. Linn. Soc.* **17**, 375–393.
- Andersson, M. 1986 Evolution of condition-dependent sex ornaments and mating preferences: sexual selection based on viability differences. *Evolution* **40**, 804–816.
- Andersson, M. 1994 *Sexual selection*. Princeton University Press.
- Darwin, C. 1871 *The descent of man, and selection in relation to sex*. London: J. Murray.
- Dumont, H. J., Mertens, J. & De Coster, W. 1993 The *Calopteryx splendens*-cline in southwestern France, analysed by quantitative wingspot analysis (Zygoptera: Calopterygidae). *Odonatologica* **22**, 345–351.
- Folstad, I. & Karter, A. J. 1992 Parasites, bright males, and the immunocompetence handicap. *Am. Nat.* **139**, 603–622.
- Getty, T. 1998 Handicap signalling: when fecundity and viability do not add up. *Anim. Behav.* **56**, 127–130.
- Grafen, A. 1990 Biological signals as handicaps. *J. Theor. Biol.* **144**, 517–546.
- Grether, G. F. 1996a Intrasexual competition alone favours a sexually dimorphic ornament in the rubyspot damselfly *Hetaerina americana*. *Evolution* **50**, 1949–1957.
- Grether, G. F. 1996b Sexual selection and survival selection on wing coloration and body size in the rubyspot damselfly *Hetaerina americana*. *Evolution* **50**, 1939–1948.
- Grether, G. F. 1997 Survival cost of an intrasexually selected ornament in a damselfly. *Proc. R. Soc. Lond. B* **264**, 207–210.
- Grether, G. F. & Grey, R. M. 1996 Novel cost of a sexually selected trait in the rubyspot damselfly *Hetaerina americana*: conspicuousness to prey. *Behav. Ecol.* **7**, 465–473.
- Gupta, A. P. (ed.) 1986 *Hemocytic and humoral immunity in arthropods*. New York: Wiley.
- Gustafsson, L., Nordling, D., Andersson, M. S., Sheldon, B. C. & Qvarnström, A. 1994 Infectious diseases, reproductive effort and the cost of reproduction in birds. *Phil. Trans. R. Soc. Lond. B* **346**, 323–331.
- Hooper, R., Tsubaki, Y. & Siva-Jothy, M. 1999 Expression of a costly, plastic secondary sexual trait is correlated with age and condition in a damselfly with two male morphs. *Physiol. Entomol.* **24**, 364–369.
- Johnstone, R. A. 1995 Sexual selection, honest advertisement and the handicap principle: reviewing the evidence. *Biol. Rev.* **70**, 1–65.
- Kimball, R. T., Ligon, J. D. & Merola-Zwartjes, M. 1997 Fluctuating asymmetry in red jungle fowl. *J. Evol. Biol.* **10**, 441–457.
- Köning, C. & Schmid-Hempel, P. 1995 Foraging activity and immunocompetence in workers of the bumble bee, *Bombus terrestris* L. *Proc. R. Soc. Lond. B* **260**, 225–227.
- Kotiaho, J. S. 2000 Testing the assumption of conditional handicap theory: costs and condition dependence of a sexually selected trait. *Behav. Ecol. Sociobiol.* **48**, 188–194.
- Krebs, C. J. 1989 *Ecological methodology*. New York: Harper & Row.
- Lagesen, K. & Folstad, I. 1998 Antler asymmetry and immunity in reindeer. *Behav. Ecol. Sociobiol.* **44**, 135–142.
- Leonard, C., Ratcliffe, N. A. & Rowley, A. F. 1985 The role of prophenoloxidase activation in non-self recognition and phagocytosis by insect blood cells. *J. Insect Physiol.* **10**, 789–799.
- Maynard Smith, J. & Harper, D. G. C. 1988 The evolution of aggression: can selection generate variability? *Phil. Trans. R. Soc. Lond. B* **319**, 557–570.
- Millar, D. A. & Ratcliffe, N. A. 1994 Invertebrates. In *Immunology: a comparative approach* (ed. R. J. Turner), pp. 29–68. Chichester, UK: Wiley.
- Møller, A. P. 1997 Developmental stability and fitness: a review. *Am. Nat.* **149**, 916–932.
- Møller, A. P. & Swaddle, J. P. 1997 *Asymmetry, developmental stability and evolution*. Oxford University Press.
- Møller, A. P., Kimball, R. T. & Erritzoe, J. 1996 Sexual ornamentation, condition, and immune defence in the house *Passer domesticus*. *Behav. Ecol. Sociobiol.* **39**, 317–322.
- Nur, N. & Hasson, O. 1984 Phenotypic plasticity and the handicap principle. *J. Theor. Biol.* **110**, 257–297.
- Palmer, A. R. 1999 Detecting publication bias in meta-analysis: a case study of fluctuating asymmetry and sexual selection. *Am. Nat.* **154**, 220–233.
- Palmer, A. R. & Strobeck, C. 1986 Fluctuating asymmetry: measurement, analysis, patterns. *A. Rev. Ecol. Syst.* **17**, 391–421.
- Pech, L. L. & Strand, M. R. 1995 Encapsulation of foreign targets by haemocytes of *Pseudoplusia includens* involves an RGD-dependent cell adhesion mechanism. *J. Insect Physiol.* **41**, 481–488.
- Plaistow, S. & Siva-Jothy, M. T. 1996 Energetic constraints and mate-securing tactics in the damselfly *Calopteryx splendens xanthostoma* (Charpentier). *Proc. R. Soc. Lond. B* **263**, 1233–1238.

- Ratcliffe, N. A. 1993 Cellular defence responses of insects: unresolved problems. In *Parasites and pathogens of insects* (ed. N. E. Berkage, S. N. Thompson & B. A. Federici), pp. 267–304. London: Academic Press.
- Rohwer, S. & Rohwer, F. C. 1978 Status signalling in Harris sparrows: experimental deceptions achieved. *Anim. Behav.* **26**, 1012–1022.
- Ryder, J. J. & Siva-Jothy, M. T. 2000 Male calling song provides a reliable signal of immune function in a cricket. *Proc. R. Soc. Lond. B* **267**, 1171–1175.
- Saino, N. & Møller, A. P. 1996 Sexual ornamentation and immuno-competence in the barn swallow. *Behav. Ecol.* **7**, 227–232.
- Schmid-Hempel, R. & Schmid-Hempel, P. 1998 Colony performance and immunocompetence of a social insect, *Bombus terrestris*, in poor and variable environments. *Funct. Ecol.* **12**, 22–30.
- Simmons, L. F., Tomkins, J. L., Kotiaho, J. S. & Hunt, J. 1999 Fluctuating paradigm. *Proc. R. Soc. Lond. B* **266**, 593–595.
- Siva-Jothy, M. T. 1999 Male wing pigmentation may affect reproductive success via female choice in a calopterygid damselfly (Zygoptera). *Behavior* **136**, 1365–1377.
- Siva-Jothy, M. T. & Plaistow, S. J. 1999 A fitness cost of eugregarine parasitism in a damselfly. *Ecol. Entomol.* **24**, 465–470.
- Siva-Jothy, M. T. & Tsubaki, Y. 1994 Sperm competition and sperm precedence in the dragonfly *Nannophya pygmaea*. *Physiol. Entomol.* **1**, 363–366.
- Siva-Jothy, M. T., Tsubaki, Y. & Hooper, R. E. 1998 Decreased immune response as a proximate cost of copulation and oviposition in a damselfly. *Physiol. Entomol.* **23**, 274–277.
- Swaddle, J. P., Witter, M. S. & Cuthill, I. C. 1994 The analysis of fluctuating asymmetry. *Anim. Behav.* **48**, 986–989.
- Thornhill, R., Møller, A. P. & Gangeastad, S. W. 1999 The biological significance of fluctuating asymmetry and sexual selection: a reply to Palmer. *Am. Nat.* **154**, 234–241.
- Valle, K. J. 1937 Geozoologisessa suhteessa mielenkiintoinen sudenkorento. *Luonnon Ystävä* **41**, 49–55.
- Valle, K. J. 1952 *Suomen eläimet 7. Sudenkorennot*. Turku: WSOY.
- Zeh, D. W. & Zeh, J. A. 1988 Condition-dependent sex ornaments and field tests of sexual selection theory. *Am. Nat.* **132**, 454–459.
- Zuk, M. & Kolluru, G. T. 1998 Exploitation of sexual signals by predators and parasitoids. *Q. Rev. Biol.* **73**, 415–438.