EFFECTS OF CELL NUMBER AND CELL SIZE ON PETIOLE LENGTH VARIATION IN A STOLONIFEROUS HERB

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In stoloniferous species, the length of petioles is of pivotal importance because it determines the position of leaf blades within the canopy. From a mechanistic perspective, two developmental processes, cell division and cell elongation, are responsible for the length of a given petiole. This study aimed at quantifying the relative contributions of cell division and cell elongation to genotypic and plastic variation in petiole length of the stoloniferous herb *Trifolium repens*. Thirty-four genotypes of *T. repens* were grown under high light conditions and simulated canopy shade. Cells were counted and their lengths measured on epidermal prints from fully grown petioles of leaves that had been initiated in the experimental light conditions. Cell number was the main trait explaining petiole length differences among genotypes grown under high light, while both cell number and length changed in response to shading. Our study revealed a strong negative correlation between shade-induced changes in cell number and cell length: genotypes that responded to shading by increasing cell numbers hardly changed in cell length, and vice versa. Our results suggest that genotypic and phenotypic variation in petiole length results from a complex interplay between the developmental processes of cell elongation and cell division.

**Key words:** cell number; cell size; petiole elongation; plasticity; shade avoidance; trade-off; *Trifolium repens*.

The interplay of genotypic differences and induced plastic responses causes plants to express differences in morphological traits (Evans and Turkington, 1988; Aarsen and Clauss, 1992; Stratton, 1995). Evolutionary processes have shaped the morphology a plant displays under a given set of environmental conditions as well as the mechanisms responsible for realizing a given phenotype (Bradshaw, 1965; Via and Lande, 1985; Sultan, 1995). The ultimate outcome of evolutionary processes depends on the relation between costs and benefits associated with the developmental processes, leading to a specific phenotype as well as costs and benefits associated with the phenotype itself (Lande and Arnold, 1983; Vantienderen, 1991; DeWitt et al., 1998; Pigliucci, 2005).

Plants can increase investment in structures that promote the acquisition of the most limiting resource (Bloom et al., 1985). For example, plants change morphologically and physiologically in response to canopy shading (i.e., elongation of stems and stem analogues, increased biomass allocation to shoots, and increased chlorophyll content [Schmitt and Wulff, 1993; Ballare et al., 1994; Stuefer and Huber, 1998; Ballare, 1999; Herault-Bron et al., 1999; Schmitt et al., 2003]) to increase resource capture under low light conditions. Elongation of vertically oriented spacers, like internodes or petioles, results in higher positioning of the light-acquiring laminas in the canopy and has therefore been argued to reduce the negative effects of shading caused by neighboring plants (Huber et al., 1998, 2004; Schmitt et al., 1999). However, elongation of structures requires increased biomechanical strength to carry the weight of the leaves and to minimize the risk of physical failure (Givnish, 2002; Anten et al., 2005; Liu et al., 2007; Huber et al., in press). Although shade-avoidance responses have a long history in plasticity research, so far it is not known how the underlying dynamic cellular processes (i.e., cell division and cell expansion) contribute to variation in trait values among genotypes and to environmentally induced variation in trait values (Smith, 2000; Sultan, 2004). This study provides new information about trait variation and plastic responses to shading at a cellular level and explores plausible evolutionary and functional consequences associated with these issues.

Plant organs such as petioles develop from one active meristem in which cell division takes place, with meristem activity determining the final cell number in the structure (Mizuikami and Fischer, 2000). Newly formed cells that no longer participate in the division process differentiate into their destined function and elongate until they reach their mature sizes (Tsukaya and Beemster, 2006). Cell division and cell elongation are distinctly different developmental processes that are separated in time and place, and as has been shown for petioles in *Arabidopsis thaliana*, different genes are independently involved in the processes regulating cell proliferation and cell elongation (Tsukaya et al., 2002). Size differences in morphological structures (i.e., petioles) can thus be achieved through differences in the total number of cells, in the size of the cells, or a combination of both. Genotypic differences in organ size or differences as a result of environmentally induced plastic responses may not have the same cellular basis, and different developmental mechanisms may contribute to genetic and plastic variations in organ size.

To date, different views exist concerning organ size control in plants (Fleming, 2002; Tsukaya, 2003). Classical cell theory states that, because cells are the basic units of a multicellular organism, the cells are the unit of organogenesis, and the final organ size is therefore primarily determined by cell number, not by cell size (Mizuikami and Fischer, 2000). This theory is supported by positive relationships found between final organ size and cell number (Bertin et al., 2003; Cookson et al., 2005). The organismal theory states that organ size is genetically determined and subject to selection, and both cell expansion and cell division can contribute to a different extent to the final organ size. This theory is supported by observations that organ size...
can, to a degree, be maintained when cell division is reduced because the effects of decreased cell number can be buffered by increased cell size (Horiguchi et al., 2006). Recently, in the context of understanding leaf morphogenesis, the neo cell theory has been proposed in which the cell is the unit of organogenesis and each cell is controlled by factors that govern the morphogenesis of which that cell (or cell population) is a part (Tsukaya, 2002). This theory suggests that a "compensatory system" is involved in leaf morphogenesis and that an increase in cell size can be triggered by a decrease in cell number and vice versa. In light of this discussion, our study will present novel results on cell size and cell number contributions to genetically determined petiole length variation expressed under common environmental conditions and in environmentally induced, plasticity increased petiole lengths.

*Trifolium repens* genotypes are highly variable in morphological traits (including petioles) when grown under identical conditions (Weijschedé et al., 2006). To our knowledge, no studies have been carried out to examine these differences at a cellular level. We previously reported that there is considerable variation in shade-induced petiole elongation among genotypes, while the absolute petiole increment due to shading was independent of the high light phenotype (Weijschedé et al., 2006). Investigating the cellular processes may increase our insight into the underlying developmental processes of the genotypic trait differences and the response to shading.

We studied the same 34 genotypes of *T. repens* as used in our previous work (Weijschedé et al., 2006) to show how cell number and cell size contribute to differences in petiole length expressed under high light conditions (genotypic differences) and to study how plasticity in cell number and cell size are involved in shade-induced petiole elongation responses. Elongation was induced in all plants by reducing the PAR and the R:FR (red:far red) ratio of the incident light. Specifically, we aimed to answer the following questions: (1) to what extent do cell number and cell size contribute to petiole length differences under high light conditions, (2) to what extent do changes in cell number and cell size contribute to shade-induced petiole elongation, and (3) how are cell number and size interrelated in shade-induced elongation?

**Materials and Methods**

**Plants and pregrowth**—*Trifolium repens*, a very common perennial herb, is known to be highly variable in morphological and developmental traits such as petiole and internode length and leaf area (Jahuf er et al., 1997). When shaded, *T. repens* shows typical shade-avoidance responses like petiole elongation and internode elongation (Solangaarachchi and Harper, 1987; Mucavitz and Turkington, 2000). Because of its stoloniferous growth form, only by adjusting the length of its petioles can *T. repens* place its laminae into upper layers of the canopy. The meristem from where a petiole develops is located directly under the base of the lamina. This site is photoreceptive and a major component in triggering the petiole elongation response (Thompson, 1995).

The 34 genotypes of *T. repens* used in this study expressed a 2–3-fold variation in petiole length under high light. In 2001, plants were collected from a single natural population on a floodplain along the River Waal near Ewijk (the Netherlands, 51°52′54″N, 5°45′00″E) and were thereafter grown under common garden conditions (Weijschedé et al., 2006). On 29 March 2004, six cuttings were made of each of the 34 genotypes. Cuttings consisted of a ramet with a well-developed root system and a lateral stolon with five rameas. These cuttings were each transferred to 0.18 × 0.22 × 0.05-m trays that were filled with a mixture of sand and sieved potting compost (2:1). To ensure sufficient nutrients throughout the experiment, we added slow release fertilizer (Osmocote Exact Mini, 3–4M, Scotts International B. V., Geldermalsen, Netherlands) to the soil mixture (4 g/L soil). Trays were filled and moistened 2 weeks prior to the beginning of planting because nutrient release starts after approximately 2 weeks.

**Experimental setup**—On 6 April 2004, plants were subjected to either homogenously shaded conditions or to control conditions, (from here on called shade and high light, respectively). The youngest visible leaf was marked at the onset of treatments. For inducing petiole elongation, plants were grown in cages covered with green transparent plastic (Lee Colortran International, An- dover, UK, no. 122, fern green), which reduced the R:FR ratio to 0.25 ± 0.01 (mean ± SE) in the cages and the PAR to 31% of the incident light. Control cages were covered with transparent plastic (Lee Colortran International, no. 120, clear), which reduced the PAR to 76% and R:FR ratio in this cage to 1.51 ± 0.02. The experiment was conducted in a heated greenhouse. Incident light was supplemented with high pressure sodium lamps (Philips GreenPower 600W, Hortilux Schréder, Monster, The Netherlands) and was 297 ± 13 μmol·m⁻²·s⁻¹ during the experiment. In a previous experiment, we showed that this setup effectively stimulated canopy shade and was sufficient to induce shade-avoidance responses (e.g., petiole elongation) and to affect plant growth (Weijschedé et al., 2006).

Treatments (shade and high light) were applied for 2 weeks and replicated in three temporal blocks (for practical reasons) with a 1-wk interval between successive blocks. Each genotype was represented once in each block × treatment combination, leading to a total of three replicates per genotype per treatment. In total, 204 plants were used for the experiment. During the experiment, plants were watered every other day with regular tap water.

**Measurements**—After 2 weeks, the first newly developed petiole, which was not visible at the onset of the experiment, was harvested and used for the measurements. In previous experiments, petioles achieved their final length in approximately 10–14 d (unpublished data). We thus assumed that leaves finished their main elongation within the 2 weeks of treatment in this experiment as well. Because in some genotypes leaf decay starts earlier in resource-poor conditions, we would not have been able to use developmentally older leaves of a comparable developmental stage across genotypes and treatments. Petiole elongation takes place in the uppermost area below the leaf blade. If petiole elongation had not finished in some of the genotype–treatment combinations, the pattern of cell length and cell number response to treatments would have differed among the different segments. However, our results showed that the qualitative results were very similar among the three segments, which further supports the notion that the petioles used in this experiment had finished development.

The length of the petiole was measured, and epidermal imprints (Schnyder et al., 1990) were made by gently laying the adaxial side of the petiole on liquid rubber (Collene President Jet Plus, Alstatten, Switzerland). The imprint functioned as a mold, and prints of the molds were made with clear nail polish. Once dried, the prints were carefully removed from the molds and put on an object glass. These prints yielded clear patterns of the upper layer of the petiole with a light microscope (Olympus BX-40, Olympus optical co., Hamburg, Germany, 200× magnification). Epidermal cells were used to represent cell number and size in the petioles (Ridge and Amarasinghe, 1984; Allard and Nelson, 1991). Three zones of the petioles, all three approximately 2 cm long, were used to assess cell number per millimeter: the top (just beneath the attachment of the laminae), the middle, and the bottom (just above the attachment of the petiole to the stolon). Within each zone, at three different randomly chosen places, cell number per millimeter was determined. Areas around stomata were not measured because these cells have markedly different sizes. Average cell number per millimeter differed per zone, but the overall response to shading did not qualitatively differ for the three zones (repeated measures ANOVA, treatment effect: F₁,₁₆₆₄ = 43.20, P < 0.001; zone of the petiole effect: F₂,₁₃₁₂ = 15.27, P < 0.001; treatment × zone: F₂,₁₆₆₄ = 0.64, P = 0.473). Total cell number per petiole was estimated as follows: the true length of each zone of the petiole (one-third of the total petiole length) was multiplied by the corresponding average number of cells per millimeter, and these three values were summed. We present cell length data (the inverse of cell number per millimeter) for the middle zone, which is the most representative zone, to show cell size variation among treatments because fully developed cells tend to be longer and wider close to the stolon but smaller and narrower close to the leaf blade (data not shown).

**Statistics**—A two-way mixed model analysis of covariance was used to test for effects of treatments, genotypes, and interactions on petiole length, cell number, and cell size. The effects of the treatments were considered fixed.
Genotypic differences under high light—Petiole length averaged 98.9 ± 3.0 mm for plants that were grown under high light, with the genotypic means ranging from 67.3 to 136.0 mm (Figs. 1A and 2). Petiole length was positively correlated with the total cell number per petiole (correlation coefficient $r = 0.821, P < 0.001$), showing that under high light longer petioles consist of more cells than do shorter petioles (Fig. 2A). Cell length did not correlate with petiole length under high light ($r = 0.110, P = 0.537$, Fig. 2B).

Overall shade effects—All plants that were moved from high light to shade responded to shading by producing longer petioles (Table 1, Fig. 1A). Petioles were on average 49% longer under shade than under high light. Total cell number increased 98.9 ± 3.0 mm for plants that were grown under high light. Petiole length was positively correlated with the total cell number per petiole (correlation coefficient $r = 0.821, P < 0.001$), showing that under high light longer petioles consist of more cells than do shorter petioles (Fig. 2A). Cell length did not correlate with petiole length under high light ($r = 0.110, P = 0.537$, Fig. 2B).

DISCUSSION
Phenotypic variation can be intrinsic (phenotype varies regardless of the environmental conditions) or plastic (phenotype varies according to environmental conditions). In this paper, we showed how the two major determinants of organ size (cell number and size) contribute to intrinsic and plastic variation of petiole length in *T. repens* under high light and shaded conditions. Our results suggest a complex relationship between the distinctly different processes that determine petiole length (cell division and cell elongation). Surprisingly, there was a high genetic variation in the relative contribution of changes in cell number and cell length to plastic petiole elongation, resulting in a trade-off in the change of cell length and cell number under shaded conditions. Because both cell elongation and cell division are associated with different costs and benefits, the relatively higher investment into one of the developmental processes is likely to have potentially large evolutionary and ecological implications.

Determinants of genotypic variation in petiole length—Genotypes of *T. repens* display a 2–3-fold variation in petiole length if grown under common garden conditions. Our study revealed that genotypic differences in petiole length can be directly related to differences in cell number: petioles that are produced under high light and that are twice as long contain on average twice as many cells. Although both cell division and cell elongation require considerable amounts of energy and carbohydrates (Voosenek et al., 2004), cell elongation is considered cheaper because this process only requires the production of extra cell wall material, whereas increasing the cell number requires additional DNA replication as well as additional cell wall material. It is thus surprising that the cost intensive process of cell division mainly contributed to genetic variation in petiole length. One possible explanation may be that biomechanical consequences associated with differences in cell length may have lead to selection against the production of longer petioles by means of increased cell expansion rather than cell division. Longer petioles need increased mechanical strength to carry the
argued to be subjected to additional mechanical forces such as relatively higher wind speed, which requires sufficient investment into organ strength (Anten et al., 2005), thereby selectively favoring investment into expensive cell division rather than into relatively inexpensive cell elongation.

Determinants of shade-induced petiole elongation—All genotypes responded to shading by elongating their petioles, and the absolute increment was independent of the petiole length under high light, confirming our earlier observation (Weijschedé et al., 2006). Much is known about the molecular basis and the signal transduction pathways of shade-induced elongation responses (Smith, 2000; Chen et al., 2004; Vandenbussche

Fig. 1. Treatment effects (mean ± SE) on (A) petiole length, (B) developmental time, (C) cell number, and (D) cell length. All characters were significantly affected by treatments (Table 1).

Fig. 2. Relation between petiole length and (A) total cell number and (B) cell length. Points show genotypic mean values. Open circles represent values under high light, closed circles represent values under shade. Significant correlations were found for petiole length and cell number under high light ($r = 0.821, P < 0.001$, solid line) and for petiole length and cell number under shade ($r = 0.788, P < 0.001$, dashed line).
et al., 2005), as well as about their ecological and evolutionary implications (Dudley and Schmitt, 1996; Schmitt et al., 1999; Weinig, 2000; Donohue et al., 2000; Callahan and Pigliucci, 2002; Huber et al., 2004). One may argue that selection will act on the response rather than on the specific cellular mechanism (cf. Calboli et al., 2003). However, the ultimate link between the molecular processes and the expression of stem length involves the control of different developmental processes (Beemster and Baskin, 1998; Tardieu et al., 2000; Francis and Sorrell, 2001; Barrero et al., 2002; Fleming, 2006; Tsukaya and Beemster, 2006). How final organ size is determined by the environment appears to be a complex mechanism which, in fact, we know very little about. The large variation in the increase in petiole lengths among genotypes, unrelated to the lengths of the high light petioles, leaves the potential for selection to act specifically on the elongation response.

On average, plastic petiole elongation was achieved by both an increase in total cell number and an increase in cell length. This result contradicts the common view that shade- and flooding-induced elongation is usually the result of cell elongation (Child et al., 1981; Reed et al., 1993; Peeters et al., 2002; Tsukaya et al., 2002; Cox et al., 2004; Kozuka et al., 2005; Voesenek et al., 2006). However, for some aquatic species variable contributions of cell division and cell elongation in flood-induced elongation have been demonstrated (Ridge and Amarasinghe, 1984; Ridge, 1987). Shade-induced changes in cell number and cell length were negatively correlated, indicating that cell division or cell expansion, two distinctly different developmental processes operating separately in space and time, act in concert to determine the given plastic petiole length increase. These results on the relative contribution of cell number and cell length can be compared with the shade-induced responses of internode length and number in determining stem height. For two Polygonum species, Griffith and Sultan (2006) found that, in contrast to our results, only the size of internodes, not the number thereof, responds plastically to shading. In contrast to our results, the lower degree of internode length plasticity in one of the species was not compensated by higher plasticity in internode number and inevitably resulted in lower height plasticity. The potential to change both cell number and cell length allows T. repens to compensate for lower plasticity in one of the traits, thereby ensuring optimal elongation.

**Developmental timing**—Much research on organogenesis has been conducted on leaf lamina development (Tardieu et al., 1999; Kaplan, 2001; Tsukaya, 2002; Aguirrezabal et al., 2006; Fleming, 2006). Laminas develop and expand as a whole to their final size while the cells divide and expand in a coordinated fashion throughout the leaf. This developmental pattern determining leaf expansion contrasts with the developmental pattern of petiole extension in T. repens. Petiole extension is achieved by cell proliferation in one meristem located at the top of petiole near the base of the laminas and subsequent cell extension within the uppermost few centimeters of the petiole (Thompson, 1995). Petiole extension is thus restricted to developmental processes within the upper part of the petiole, while the cells in the lower part of the petioles have already reached their final shape. Thus, each part of the petiole may develop at a different time during which cell proliferation and cell extension can respond to environmental triggers, enabling petioles to fine-tune their final length. In contrast to expansion of leaf lamina, expansion processes in petioles are thus not coordinated.

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**Table 1.** Results (F values and their significances) of mixed-model analysis of covariance of the effects of treatments, genotypes, and blocks on petiole length, developmental time (days needed to produce one petiole), cell number, and cell length. Significances are as follows: ns, P > 0.10; †, 0.10 < P < 0.05; *0.01 < P < 0.05; **0.05 < P < 0.01; ***P < 0.001.

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>Petiole length</th>
<th>Developmental time</th>
<th>Cell no.</th>
<th>Cell length</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>1</td>
<td>236.0***</td>
<td>21.60***</td>
<td>62.3***</td>
<td>97.6***</td>
</tr>
<tr>
<td>Genotype</td>
<td>33</td>
<td>5.3***</td>
<td>1.4 ns</td>
<td>7.2***</td>
<td>3.9***</td>
</tr>
<tr>
<td>Treatment × genotype</td>
<td>33</td>
<td>1.1 ns</td>
<td>1.5 †</td>
<td>1.1 ns</td>
<td>0.9 ns</td>
</tr>
<tr>
<td>Block</td>
<td>2</td>
<td>1.1 ns</td>
<td>0.7 ns</td>
<td>2.1 ns</td>
<td>10.1***</td>
</tr>
<tr>
<td>Error</td>
<td>134</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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Fig. 3. Relation between petiole lengths under high light (x-axes) and (A) absolute petiole length increase, (B) absolute cell number increase, and (C) absolute cell length increase. The dashed line indicates a marginally significant correlation between petiole length under high light and absolute increase in cell number in response to shading (r = −0.331, P = 0.056). Increases in petiole length, cell number, and cell length were calculated as absolute differences of petiole length, cell number, or cell length under shade and high light. Points represent genotypic means.
as high light is reached, we do not know yet which triggers determine the halt of cell proliferation and expansion in homogeneous light conditions. A possible trigger may be resource shortage, but further research is needed to answer this question.

**Interrelationship of cell number, cell size, and organ size**—
We used multiple genotypes grown under identical conditions and showed that, under high light, longer petioles consist of more cells rather than longer cells. These data are consistent with the classical cell theory stating that final organ size is determined primarily by cell number (Mizukami and Fischer, 2000; Bertin et al., 2003; Tsukaya, 2003; Cookson and Granier, 2006). Differences in petiole length in *T. repens* thus appear to have evolved via selection on the correlation between organ size and cell number. When petioles elongate in response to shading, both size and number of cells contributed to the total petiole plasticity, and small contributions (or even a reduction)

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**Table 2.** Results of a multiple regression analysis testing the extent to which the shade-induced increment in petiole length was determined by the increase in cell number and cell length.

<table>
<thead>
<tr>
<th>Increment</th>
<th>df</th>
<th>Estimate</th>
<th>SE</th>
<th>t</th>
<th>P &gt;</th>
<th>f</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>1</td>
<td>0.246</td>
<td>0.059</td>
<td>4.17</td>
<td>0.0002</td>
<td></td>
</tr>
<tr>
<td>Cell no. increase</td>
<td>1</td>
<td>0.151</td>
<td>0.059</td>
<td>2.57</td>
<td>0.0153</td>
<td></td>
</tr>
<tr>
<td>Cell length increase</td>
<td>1</td>
<td>0.151</td>
<td>0.059</td>
<td>2.57</td>
<td>0.0153</td>
<td></td>
</tr>
</tbody>
</table>

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**Fig. 4.** (A) Relation between relative change in total cell number (y-axes) and cell length (x-axes) in response to shading. Correlation (solid straight line) was calculated with all data points ($r = -0.380$, $P = 0.027$) and with all data except data point (1) ($r = -0.539$, $P = 0.001$). (B) and (C) show the relations between relative increase in petiole length and relative change in cell number ($r = 0.179$, $P = 0.329$) and cell size in response to shading ($r = 0.179$, $P = 0.329$). Dots represent genotypic means.
of one factor was buffered by an increased contribution of the other factor. These results are in line with the organismal theory stating that size (or in this case, the response) is genetically determined and subject to selection, and both cell expansion and cell proliferation can contribute to a different extent to the final size (Hemerly et al., 1995; Kaplan, 2001). The negative correlation between changes in cell number and cell length further suggests that a compensatory system operated beyond the cellular level to ensure sufficient elongation. This correlation is in line with the neo cell theory, which suggests that a “compensatory system” is involved in leaf morphogenesis and that an increase in cell size can be triggered by a decrease in cell number and vice versa (Tsukaya, 2002).

Cell number and cell length might have different functions that were selected for to different extents in different genotypes. The genotypes used in this study originate from a Dutch floodplain grassland characterized by high temporal and spatial environmental heterogeneity and species composition (van Eck et al., 2004; Voesenek et al., 2004). The herbaceous vegetation is in fact composed of a dynamic mosaic of different microhabitats, and each single clone of the stoloniferous species T. repens may experience different environmental conditions in space and in time. The most prevalent microhabitat conditions experienced by a clone may be one of the forces selecting greater responsiveness of either cell proliferation or elongation. Relatively sparse microhabitats might favor responsiveness in cell number because this character can preserve cell density and thus petiole constructive stiffness (Huber et al., in press). On the other hand, genotypes originating from more dense microhabitats might have greater plasticity in cell elongation, which may be less costly in a dense canopy where leaves can lean on their neighbors and do not depend on the rigidity of their own petioles for preventing physical failure. This study shows that variation in petiole length of T. repens results from a complex interplay between different developmental processes. Further investigation of the costs and constraints involved with these developmental processes as well as their ultimate effects on plant performance under different environmental settings will enhance our understanding of how selection operates in shaping trait characters under various environmental conditions.

LITERATURE CITED


