

# Flowering responses of serradella (*Ornithopus* spp.) and subterranean clover (*Trifolium subterraneum* L.) to vernalisation and photoperiod and their role in maturity type determination and flowering date stability

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## ABSTRACT

**Context.** Serradellas (*Ornithopus* spp.) are promising alternative annual legumes to subterranean clover (*Trifolium subterraneum* L.), for permanent, temperate pastures. However, many cultivars exhibit unstable flowering dates across years. This is a risk for seed production and persistence.

**Aim.** This study assessed how vernalisation and photoperiod cues determine maturity type and flowering date stability among serradella cultivars. **Methods.** First flower appearance was recorded for early and late maturing cultivars of yellow serradella (*Ornithopus compressus* L.), French serradella (*Ornithopus sativus* Brot.) and subterranean clover after exposure to six vernalisation treatments (0, 1, 3, 5, 7 or 9 weeks at 5°C) with subsequent growth under four photoperiods (8, 12, 16 or 20 h).

**Key results.** ‘Intrinsic earliness’ differed by only zero to three nodes for cultivars within species, indicating that maturity type was determined primarily by a cultivar’s responses to vernalisation and photoperiod. An interaction between these responses was observed, with a precipitous decline in the requirement for vernalisation when photoperiods exceeded 12 h. Many cultivars also displayed a persistent component to their vernalisation response, whereby long photoperiods (20 h) did not completely negate the response to vernalisation. **Conclusions.** Later maturity was associated particularly with need for long exposure to the vernalisation treatment to minimise the duration from sowing to first flower appearance. Stable flowering is more likely when a cultivar has components of its vernalisation requirement that are not satisfied before autumn ends to prevent premature flowering, and a photoperiod response in spring that overrides any unmet vernalisation requirement. **Implications.** Persistence by serradella cultivars requires selection for suitable responsiveness to vernalisation and photoperiod.

**Keywords:** adaptation, annual legumes, daylength, Mediterranean, pasture, persistence, phenology, temperate.

## Introduction

Subterranean clover (*Trifolium subterraneum* L.) is the dominant legume used in grass–legume pastures on acid soils across southern Australia (Nichols *et al.* 2012). A number of alternative annual pasture legumes have been trialled over many years in an attempt to diversify the legume base of these pastures (Howieson 1993; Howieson *et al.* 2000; Loi *et al.* 2005; Nichols *et al.* 2007). However, only a few have proven to have potential for wide adoption, including: biserrula (*Biserrula pelecinus* L.), yellow serradella (*Ornithopus compressus* L.) and French serradella (*Ornithopus sativus* Brot.) (Hill 1996; Nichols *et al.* 2012). The serradellas have potential suitability over a similar, if not larger, climatic adaptation zone to that of subterranean clover (Hill 1996). They are well-adapted to acid soils (pH<sub>Ca</sub> 3.9–7.0, Michalk 1993; Guo *et al.* 2012) and have achieved herbage yields that mirror or exceed that of subterranean clover (Bolland 1987; Freebairn *et al.* 1997; Dear *et al.* 2002; Hayes *et al.* 2017; Sandral *et al.* 2019).

Despite these attributes, serradellas have mainly been used in short pasture phases (1–5 years) of crop rotations on acidic sandy soils in low–medium rainfall zones (e.g. southern Western Australia and northern NSW, [Bolland and Gladstones 1987](#); [Oram 1987](#); [Freebairn 1990](#)) and, more recently, on acidic, loamy and duplex soils in southern NSW ([Dear \*et al.\* 2004](#); [Hackney \*et al.\* 2021](#)). Yellow serradellas, in particular, have also been used as the legume base for longer-term (permanent) pastures on deep, sandy soils in Western Australia and northern NSW. For example, at Coonabarabran in northern NSW, the cultivar ‘King’ is reported to maintain high plant densities in pasture swards for at least 15 years ([Freebairn 1990](#)). A wider role for yellow and French serradellas in permanent pastures of the temperate, high rainfall (>700 mm annual rainfall) zone of south-eastern Australia has been advocated ([Sandral \*et al.\* 2019](#)). However, long-term persistence by serradellas is a prerequisite for their adoption. Subterranean clover cultivars are typically expected to persist at high plant densities for up to 20–25 years because of the major cost of pasture renovation ([Scott \*et al.\* 2000](#); [Virgona and Hildebrand 2006](#)). Longer term experiments (>3 years) with serradellas are rare. Where this has occurred, the ability of a number of the current serradella cultivars to persist in high rainfall pastures has been questioned ([Hayes \*et al.\* 2015, 2023](#)).

Long-term persistence by pasture species can depend on a number of factors including: grazing tolerance, acid soil and toxic aluminium tolerance and disease resistance ([Reeve \*et al.\* 2000](#); [Virgona and Hildebrand 2006](#); [Hayes \*et al.\* 2019](#)). However, the primary persistence traits of annual pasture species are related to reliable flowering, high seed set and seed protection mechanisms ([Loi \*et al.\* 2005](#); [Taylor 2005](#); [Teixeira \*et al.\* 2022](#)), which ensure regeneration in subsequent growing seasons ([Donald 1970](#); [Dear \*et al.\* 2002](#); [Guo \*et al.\* 2022](#)). Persistence by serradellas in new environments requires flowering at an optimal time. Typically, annual species must flower during spring at a time that avoids the risk of frost that damages flowers and developing seeds, with sufficient time remaining for seed production before the onset of heat and drought stresses that terminate seed filling ([Aitken 1974](#); [Flohr \*et al.\* 2017](#); [Lilley \*et al.\* 2019](#); [Lake \*et al.\* 2021](#)).

The ideal flowering date for seed production is characteristic of each local environment, and is achieved by matching cultivar maturity type (usually defined by flowering date rankings) to the local growing season environment ([Aitken 1974](#); [Nichols \*et al.\* 2013](#)). This strategy also maximises animal feed production in each growing season environment ([Thomas \*et al.\* 2021](#)). In southern Australia, the ideal flowering date occurs between late winter (August) in low rainfall Mediterranean environments (250–500 mm) where growing season length is short (3.5–5 months), and late spring (November) in higher rainfall (700–900 mm per annum), cool temperate environments that have longer growing seasons (e.g. 7.5–9 months). A complication for regular achievement of the ideal flowering date is the timing of the opening seasonal rainfall, and thus the date of germination.

This is highly variable in southern Australia. Opening seasonal rainfall sufficient to germinate seeds may occur at any time between February and June within the south-eastern Australian pasture zone ([Clark \*et al.\* 2003](#); [Pook \*et al.\* 2009](#)). Annual pasture legumes need to flower at their ideal date each year irrespective of their germination date (hereafter we term this characteristic: ‘stable flowering’). In addition to the normal interannual variation in the timing of opening seasonal rainfall, recent comparison of climate data for 1990–2018 with that of the 1971–1989 period in southern Australia indicates that for some regions, the median date of the break of season has advanced by up to 17 days, whilst delays of up to 11 days have occurred elsewhere ([Flohr \*et al.\* 2021](#)). Climate change that may shift the timing of germination over the expected life (20–25 years) of a permanent pasture, further increases the need for cultivars with stable flowering dates.

Stable flowering is a characteristic of many subterranean clover cultivars, when grown in the districts for which they are recommended (e.g. [Boschma \*et al.\* 2019](#)), but it is not a characteristic of many of the serradella cultivars currently available in southern Australia ([Boschma \*et al.\* 2019](#); [Haling \*et al.\* 2023](#)). However, [Boschma \*et al.\* \(2019\)](#), found that late maturing serradella cultivars tended to display more stable flowering than early and mid-season cultivars.

The date on which a plant flowers at a field location is determined by thermal time accumulation at that location and its impact (1) on the expression of the ‘intrinsic earliness’ of the plant genotype as modified by its (2) vernalisation and/or (3) photoperiod requirements for flowering ([Hochman 1987](#); [Evans \*et al.\* 1992](#); [Liu 2007](#); [Teixeira \*et al.\* 2020](#)). We use the term ‘intrinsic earliness’ to represent a cultivar’s earliest thermal time (and lowest node) at which flowering is observed. This cultivar characteristic is observed when fully-vernalised seedlings are grown under inducing photoperiods that satisfy the cultivar’s photoperiod requirement ([Slafer 1996](#)). These requirements and intrinsic earliness of various subterranean clover cultivars are well studied ([Aitken 1955, 1974](#); [Evans 1959](#); [Collins and Smith 1975](#); [Salisbury \*et al.\* 1987](#); [Teixeira \*et al.\* 2021](#)) but there is only limited information concerning aspects of the response to photoperiod and vernalisation available for cultivars of yellow or French serradella ([Gladstones and Devitt 1971](#); [De Ruiter and Taylor 1979](#); [Fu \*et al.\* 1994](#); [Nutt 2010](#)). We hypothesised that differences in flowering date among early and late serradella cultivars ([Boschma \*et al.\* 2019](#)) would be attributable to differences in their response to vernalisation and photoperiod stimuli, and that later maturing types were likely to require longer exposure to vernalising temperatures, or longer photoperiods to reduce their time to flower. It was anticipated that an understanding of the interaction and magnitude of vernalisation and photoperiod responses among cultivars would reveal characteristics important for stable flowering. A controlled-environment study was conducted to examine the flowering responses of serradella to photoperiod and vernalising

temperature cues. Early and late maturing yellow serradella and French serradella cultivars were compared with early and late subterranean clover cultivar controls.

## Materials and methods

### Plant material

The experiment utilised two cultivars of three pasture legume species. Early and late cultivars of yellow serradella were 'King' and 'Avila', French serradella cultivars were 'Eliza' and 'Serratas' and subterranean clover cultivars were 'Izmir' and 'Goulburn', respectively.

### Plant growth conditions and the imposition of treatments

#### Sowing and establishment

Seeds of yellow serradella cultivars were hard (impermeable to water) and were scarified by rubbing seeds gently between sheets of fine (180-grit) sandpaper (10 passes back and forth). Seed lots with verified rapid germination were then used to establish the treatments. French serradella and subterranean clover seeds were soft and did not require any prior preparation.

Seeds were sown in pots containing 2 kg of a nutrient-rich, pasteurised, sandy-loam soil to establish two uniform seedlings per pot after thinning. Each pot represented one replicate and, whilst both plants were grown for the duration of the experiment, observations were only collected from the first plant to flower. The soil was moistened and the pots were covered with black plastic and kept at 4°C (soil temperature) for 48 h to minimise impacts of embryo dormancy and to promote even germination.

The plants were then transferred to a growth cabinet fitted with fluorescent and incandescent lighting (480  $\mu\text{mol photons/m}^2\text{s}$ , visible range) and set to 8 h light (21°C)/16 h dark (17°C) (i.e. conditions assumed to be non photoperiod- and non vernalisation-inducing) to allow seedlings to establish for 14 days.

#### Vernalisation and photoperiod treatments

The experiment was conducted using a complete factorial combination of six cultivars  $\times$  six vernalisation treatments  $\times$  four photoperiod treatments  $\times$  six replicates (Supplementary Table S1). At 16 days after sowing when the unifoliate leaf was visible in subterranean clovers (V2 stage, Teixeira *et al.* 2021) or the first pinnate leaf of the serradellas was visible, established cohorts of the six cultivars were subjected to one of six vernalisation (VRN) treatments (0, 1, 3, 5, 7 or 9 weeks, with 8 h photoperiod). The vernalisation temperature was measured as close as practical to the growing point position of the plants and fluctuated between the lights on (8°C for 8 h) and off (4°C for 16 h) periods. All thermal time calculations assume a weighted average temperature

of 5°C for each day in the vernalisation treatment. Sowing and, consequently, entry to each of the VRN treatments was timed, using a staggered schedule, so that all plants exited the VRN treatments on the same day.

Plants were then subjected to one of four photoperiod (PPD) treatments (8, 12, 16 or 20 h photoperiod). The PPD treatments were conducted in matched plant growth cabinets fitted with fluorescent and incandescent lighting (480  $\mu\text{mol photons/m}^2\text{s}$ , visible range) that were used for 8 h at a temperature of 21°C. For the remainder of each day (16 h), the temperature was 17°C. Extended photoperiods (12, 16 and 20 h) were achieved by only using the incandescent lighting (18  $\mu\text{mol photons/m}^2\text{s}$ ) for time periods that were also centred around the midpoint of the daily 8 h period of full light. When plants entered PPD treatments, a plastic mesh (10 mm  $\times$  10 mm) was fitted to the outside of their pots so that leaf canopies were contained but not shaded.

#### General management of plant growth

Plants were monitored regularly and managed to avoid potential confounding effects of inadequate nutrient supply, insect pests or plant disease issues. Pots were watered daily to 80% field capacity and once each week to 100% field capacity to ensure that plants were neither waterlogged nor water stressed. Nitrogen fixation was ensured by inoculating the plants 9 days after sowing with a species-specific *Rhizobium* strain: i.e. *Bradyrhizobium lupini* (WSM471; New Edge Microbials, Albury) and *Rhizobium leguminosarum* bv. *trifolii* (WSM1325) for the serradellas and subterranean clovers, respectively. Nutrient solution (Aquasol [Yates, Clayton], N:P:K [23:4:1 w/w/w] plus micronutrients) was prepared at 8 g Aquasol per 5 L of water and 60 mL of solution was added to all pots at 2-monthly intervals.

Later flowering treatments (8 and 12 h PPD) were treated with fungicide (500 g/L spiroxamine, Bayer, Pymble) as a dilute spray of 60 mL/100 L, 6 and 8 months after sowing the zero VRN treatments, due to early signs of powdery mildew appearing on leaves. However, canopies of some replicates of treatments where flowering was most delayed, became so dense that leaf disease could not be controlled and these plants were removed from the experiment. Consequently, data for only three of six original replicates were obtained for the following treatments: 'Avila', 8 h PPD (3- and 5-week VRN), 12 h PPD (0, 5- and 7-week VRN); 'Goulburn', 8 h PPD (3-week VRN), 12 h PPD (0 VRN). Additionally, only one replicate was obtained for 'Goulburn' in the 8 h PPD (0 VRN) and no data were obtained for 'Goulburn' in the 8 h PPD (1-week VRN) treatment (Table S1).

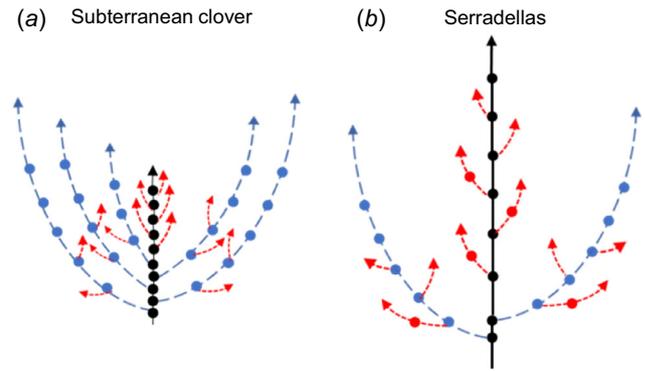
#### Node of first flower

The node at which the first flower appeared was recorded as a measure of the 'duration' of the vegetative phase (germination to floral initiation). This measure is independent of rate of node production. The convention of counting the cotyledonary

node as zero was adopted (Evans 1959; Aitken 1974; Collins and Wilson 1974; Salisbury *et al.* 1987) and node one was thus the site at which the first leaf appeared for all species. The node of first flower, recorded when plants were grown in the longest VRN (9 weeks) and PPD (20 h) treatments, was considered to be a reasonable representation of the minimum node of first flower and a cultivar's 'intrinsic earliness'; the proviso being that seedlings were established, for 16 days after sowing, under conditions unlikely to induce photoperiod or vernalisation responses. Plants developed one leaf during this 16 day period. This was followed by a vernalisation treatment under an 8 h photoperiod during which node development will have continued albeit at a slow rate. Our measure of intrinsic earliness is not corrected for the number of nodes produced under the vernalisation treatment because insufficient data were collected to make such a correction. Node numbers greater than the minimum node of first flower measured in the VRN-9 weeks and PPD-20 h treatments were interpreted as indicating a delay to flowering and that vernalisation and/or photoperiod requirements of the cultivar had not been fully satisfied by a treatment.

### Description of shoot morphology

There are various terms used to describe branching patterns in different pasture legumes. Much of the subterranean clover literature describes the formation of nodes firstly on a main stem followed by branching in which new axes, referred to as 'runners', are formed and from which subsequent axes, 'laterals', can form (Aitken and Drake 1941; Teixeira *et al.* 2021). Different terminology has been used to describe the architecture of serradella plants. For example, the first level of stem branching has been described as the 'primary branches' (Revell 1997) or 'primary stems' (Nutt 2010). For uniformity within and among species descriptions, we adopted the description of legume shoot morphology proposed by Faverjon *et al.* (2017), which uses a universal terminology to compare legumes with differing morphology. Stems and branches are referred to as axes and were denoted: main > primary > secondary > higher order axes, according to their order of appearance (Fig. 1). The basic structure of subterranean clover and serradellas is a singular main axis from which major primary axes form (Fig. 1). They are 'crown-forming' legumes (after Faverjon *et al.* 2017) in which the main axis continues to form nodes and elongates (serradellas) or continues to form nodes but does not elongate (subterranean clover) as the plant grows. Subterranean clover differs from the serradella species in the number of major primary axes that are formed. These major axes become co-dominant stems alongside the main stem. Subterranean clover may have 5–7 of these primary axes (i.e. the 'basal runners' as described by Aitken and Drake (1941)) and serradella species frequently have two primary axes (i.e. 'primary branches' as described by Revell (1997)). Axes arising from these



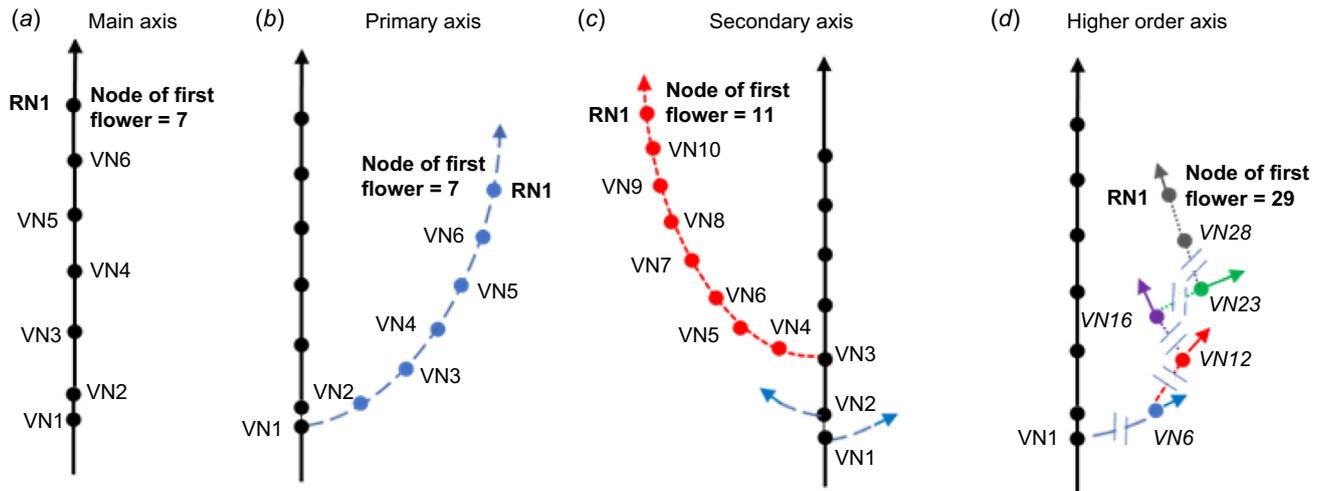
**Fig. 1.** Typical node development (circles denote nodes) of crown-forming plants: subterranean clover (main axis does not elongate) (a), French or yellow serradella species (main axis does elongate) (b). Main axis (black solid line), primary axes (blue, long dashed lines) and secondary axes (red, short dashed lines). First flowers most frequently occurred on main or primary axes, except when flowering was very delayed. First flowers then appeared on secondary or higher order axes (see Fig. 2).

primary axes and the main axis, as it continues to grow, were referred to as secondary axes. Higher order axes, if formed, occurred subsequently on secondary, tertiary, etc. axes.

With this in mind, we determined node of first flower by counting the number of vegetative nodes from node one to the node at which the first flower appeared (i.e. the first reproductive node, RN1). For example, where the node of first flower was on the main axis, nodes were simply counted along the main axis. Alternatively, where the node of first flower was positioned on a primary, secondary, or higher order axis, node counts included nodes that appeared on the pathway to the node of first flower (Fig. 2).

### Thermal time to flower

Thermal time to flower was used as an additional measure of the duration to flowering. Thermal time was calculated using weighted mean daily temperatures that accounted for the time that plants were growing under the maximum (day time) and minimum (night time) temperatures in the controlled environment and vernalisation chambers. A linear response between a base temperature of 0°C and an optimum temperature of 21°C was assumed for all of the genotypes based on mean cardinal temperatures reported for subterranean clover (Moot *et al.* 2000; Monks *et al.* 2009). The sowing depth was shallow (1–2 mm) and, therefore, the time between sowing and emergence was short (5–7 days) and consistent across treatments. Consequently, thermal time accrual started at sowing and ended at the appearance of the first flower (petals emerging from the calyx by 2–3 mm). Thermal time used in this way allowed us to equate growth in the cooler vernalisation treatment with that in the warmer temperatures of the photoperiod treatments.



**Fig. 2.** Examples of how nodes were counted when the first flower appeared on: main (a), primary (b), secondary (c) or higher order axis (d) of the serradellas. Vegetative nodes (VN) indicate node progression along each axis (NB: not all nodes are shown on the higher order axis schematic). First reproductive node (RN1). First flowers only appeared occasionally on the higher order axes of serradella cultivars with substantially delayed flowering. The highest order axis recorded for RN1 was a fifth order axis (as shown in this schematic).

## Statistical analysis

The experiment was designed as a three-way factorial completely randomised design, with six replicates of the six cultivars  $\times$  six VRN treatments  $\times$  four PPD treatments. The response variable, ‘time to flower’ (node of first flower and thermal time to first flower) was analysed using a three-way factorial ANOVA analysis using the ‘lm’ function in R v4.0.5 (R Core Team 2021), with the ‘predictmeans’ package (Luo *et al.* 2021) used to obtain predicted means, standard errors and 5% least significant differences (l.s.d.) for pairwise comparisons of means. The response variable was log-transformed prior to analysis to ensure homogeneity of variance and approximate normality of the analysis residuals, and so analysis results were obtained, and post-analysis inference conducted, on the log-transformed scale. The blocking in the design was found to have no impact on the ANOVA result and was therefore not included in the final statistical analysis. The data points shown in figures are back-transformed means of the  $\log_{10}$  data from the ANOVA. The error bars shown on each point are back-transformed l.s.d. bars based on the 3-way interaction l.s.d. ( $P = 0.05$ ). Where the bars for two means did not overlap, the means were regarded as significantly different according to Fisher’s 5% l.s.d. test.

## Results

### Statistical analysis summary

The results of the ANOVA revealed a highly significant ( $P < 0.001$ ) three-way interaction term (cultivar  $\times$  VRN  $\times$  PPD) for analysis of both response terms (node of first flower and thermal time to first flower), with average 5% Fischer’s l.s.d. values of 0.09 ( $\log_{10}(\text{node of first flower})$ ) and 0.08

( $\log_{10}(\text{thermal time to first flower})$ ) were obtained from the analysis.

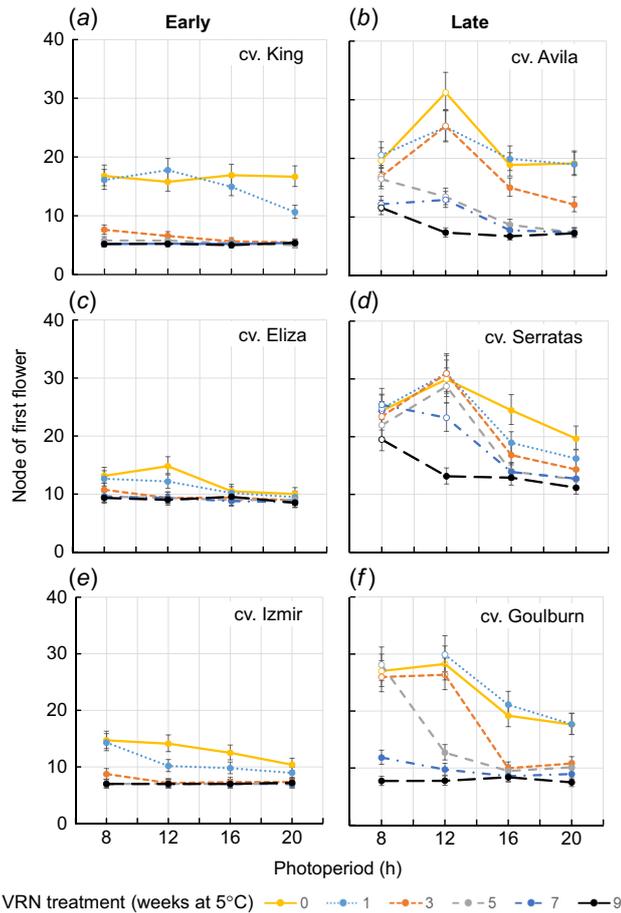
### Intrinsic earliness

We used the maximum VRN (9 weeks) and PPD (20 h photoperiod) treatments to define minimum node of first flower (Fig. 3). However, it was in fact achieved by all cultivars in the 9-week VRN treatment provided they were grown with at least a 12 h PPD. Minimum node of first flower ranged from 5 to 11 nodes among the cultivars: King (5), Avila (7), Eliza (8), Serratas (11), Izmir (7) and Goulburn (7). The minimum flowering node was not different for early and late maturing subterranean clover cultivars. However, within each serradella species, the minimum node of first flower of the early maturing cultivars (‘King’ and ‘Eliza’; 5 and 8, respectively) were lower than those of the late maturing cultivars (‘Avila’ and ‘Serratas’; 7 and 11) ( $P < 0.001$ ). Nevertheless, the differences were small relative to the much larger number of nodes at which first flowers appeared when vernalisation and photoperiod requirements had not been met.

### Response to VRN and PPD treatments

Flowering occurred in all treatments and the cultivars exhibited responses typical of ‘quantitative long day’ plants with ‘facultative’ vernalisation responses (i.e. vernalisation was not essential for flowering to occur, but flowering occurred more rapidly after vernalisation had been experienced) (Thomas and Vince-Prue 1997).

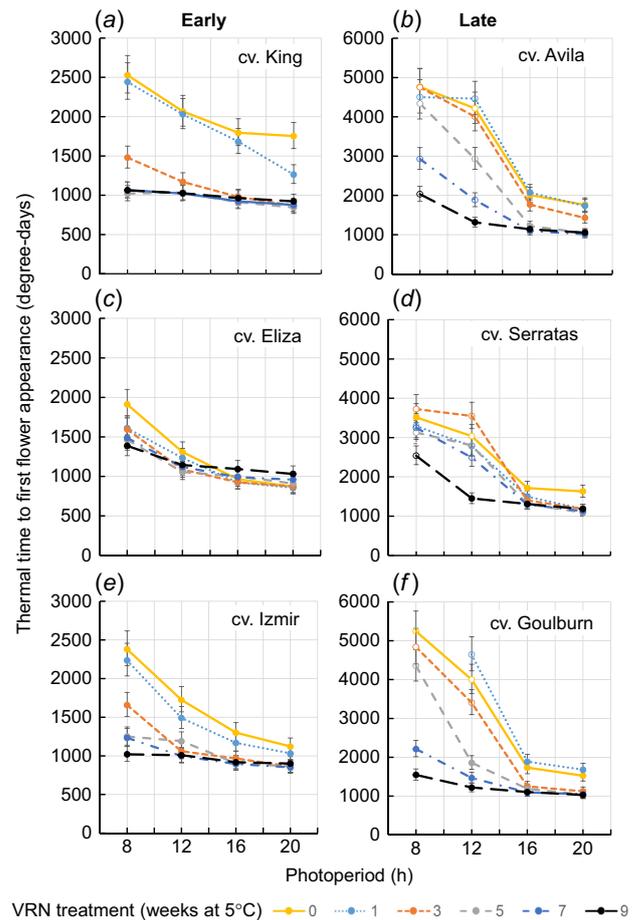
Flowering was usually delayed when the duration of vernalisation and photoperiod stimuli were decreased, leading to an increase in the number of nodes developed before the appearance of the first flowers. However, the largest node



**Fig. 3.** Node of first flower for early and late maturing yellow serradella (a, b), French serradella (c, d) and subterranean clover cultivars (e, f) in response to VRN and PPD treatments. The data points are back-transformed means of the  $\log_{10}$  data used for ANOVA. The error bars shown on each point are back-transformed I.s.d. bars based on the 3-way interaction I.s.d. ( $P = 0.05$ ). Where the bars for two means do not overlap, the means may be regarded as significantly different according to Fisher's 5% I.s.d. test. Open symbols denote data where first flowers, appeared on secondary or higher order axes.

of first flower (31) was observed on unvernalsed, late maturing cultivars grown under the 12 h PPD. The anticipated trend to increased node numbers at lower photoperiods was broken when the late maturing cultivars were grown with an 8 h PPD because the node of first flower in this PPD treatment was either equal to, or often less than, that counted in the 12 h PPD treatment. This observation was associated invariably with very late flowering times (i.e. Fig. 4) and with flowering at a node on a secondary or higher order axis.

First flowers appeared at the 13th–16th nodes of the early maturing cultivars, also indicating some delay in flowering time in the absence of vernalisation and/or photoperiod stimuli (Fig. 3). Nevertheless, it was clear by comparison with the late maturing cultivars, that the early cultivars expressed



**Fig. 4.** Thermal time to first flower appearance (degree-days) for early and late maturing yellow serradella (a, b), French serradella (c, d) and subterranean clover cultivars (e, f) in response to VRN and PPD treatment. The data points are back-transformed means of the  $\log_{10}$  data used for ANOVA. The error bars shown on each point are back-transformed I.s.d. bars based on the 3-way interaction I.s.d. ( $P = 0.05$ ). Where the bars for two means do not overlap, the means may be regarded as significantly different according to Fisher's 5% I.s.d. test. Open symbols denote data where first flowers appeared on secondary or higher order axes.

very low to negligible responses to photoperiod and had low ('King', 'Izmir') or negligible ('Eliza') requirements for vernalisation to hasten flowering. The largest delay in flowering (i.e. up to 16 nodes) observed among the early cultivars occurred for 'King'. This cultivar exhibited a relatively small requirement for at least 1 week of the VRN treatment before flowering was hastened (Figs 3–5).

### Axis of first flower

The first flowering node was often located on the main or primary axis, especially in treatments where there was a short delay to flowering. This included all VRN × PPD

treatments for early maturing cultivars, as well as all 16 and 20 h PPD (regardless of VRN) treatments and 9-week VRN (regardless of PPD) treatments for late developing cultivars (Figs 3, 4). First flowers began to appear on secondary or higher order axes in the remaining late developing cultivar treatments, i.e.  $\leq 12$  h PPD,  $< 9$ -week VRN. However, flowering on higher axis orders was only observed when flowering was most delayed. The highest order axis on which a first flower was observed was a fifth order axis, but this only occurred on two plants (e.g. Fig. 2).

### Interactions between VRN and PPD responses

Among the late maturing cultivars, there was a distinct interaction between the flowering node response to the VRN and PPD treatments ( $P < 0.001$ ) (Fig. 3). This was most clearly indicated by the substantially lower number of nodes to first flower observed when plants were grown in the 16 h PPD, as compared to growth in the 12 h PPD treatment, after the cultivars had experienced a low VRN stimulus (e.g. 1–3 weeks). Effectively, growth in longer photoperiods ( $> 16$  h) substituted much of the need for vernalisation to hasten flowering.

Likewise, longer VRN periods (e.g.  $> 3$  weeks for ‘Goulburn’, or  $> 7$  weeks for ‘Avila’ and ‘Serratas’) substituted the need for long PPD treatments ( $> 16$  h) to hasten flowering (Fig. 3). For four of the six cultivars, the longest VRN treatment completely overrode the need for any photoperiod stimulus to ensure flowering at the minimum node. The exceptions were the late maturing serradella cultivars, ‘Avila’ and ‘Serratas’, which required either a much longer VRN treatment than was applied in this experiment, or a photoperiod of at least 12 h combined with the 9-week VRN treatment to flower at the minimum node.

There were fewer interactions evident between the photoperiod and vernalisation responses of the early maturing cultivars. However, the generally low or negligible responses to the PPD and/or VRN treatments by the early cultivars inevitably precluded the expression of such interactions. Where an interaction was observed (e.g. ‘Izmir’), the node number response to VRN and/or PPD was typically fewer than five nodes.

### Persistent vernalisation requirement

Despite clear evidence of an interaction between the responses to PPD and VRN ( $P < 0.001$ ), all cultivars except ‘Eliza’, retained a need for some vernalisation to hasten flowering when grown with very long days. Thus, there was often a ‘persistent requirement’ for vernalisation (defined as the difference in time to flower responses observed between 0 VRN-20 h PPD and 9 week VRN-20 h PPD treatments), which was not eliminated when grown in the 20 h PPD treatment. If the persistent vernalisation requirement was not met, flowering in ‘King’ and the late

maturing cultivars (‘Avila’, ‘Serratas’ and ‘Goulburn’) was delayed by 8–12 nodes (Fig. 3). For ‘Izmir’ the flowering delay was  $\sim 3$  nodes. The length of VRN treatment required to completely overcome the persistent vernalisation requirement was relatively small for the affected early cultivars (1–3 weeks VRN; ‘King’ and ‘Izmir’), but was considerable for the late cultivars (5–7 weeks VRN).

### Thermal time to flower

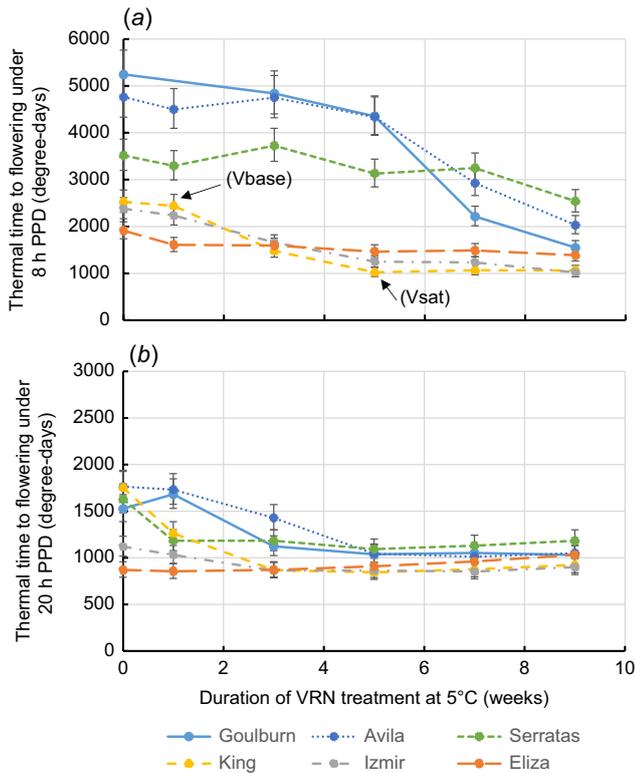
The long thermal times from sowing to first flower observed for late maturing cultivars demonstrated that they required exposure to longer PPD and VRN treatments to stimulate earlier flowering in comparison with the earlier maturing cultivars (Fig. 4). A need for longer VRN to minimise time to flower under the shortest PPD treatment (8 h) was now also indicated; this was not obvious when using only the node of first flower observations (Fig. 3). Thermal time to flower demonstrated unequivocally that the longest delays to flowering for all cultivars were observed under the ‘8 h PPD-0 VRN’ treatment, although four of the cultivars also achieved, or came close to achieving, their longest time to first flower under 12 h photoperiods (e.g. ‘Serratas’). Early maturing cultivars were the least delayed, with their longest thermal times to flower being: Eliza, 1912 degree-days (i.e.  $^{\circ}\text{C days}$ ); Izmir, 2380 degree-days and King, 2528 degree-days. In the absence of vernalisation or photoperiod stimuli, late maturity cultivars exhibited substantially longer maximum thermal times to flower: Serratas, 3515 degree-days; Avila, 4763 degree-days and Goulburn, 5244 degree-days.

In the late maturing cultivars, a distinct interaction between the responses to VRN and PPD was also observed when using thermal time to flower. For these cultivars, increasing the amount of either VRN or PPD tended to override the requirement for PPD or VRN stimuli, respectively (Fig. 4). The persistent requirement for vernalisation, noted above, was also indicated by thermal time to flower in the longest PPD (20 h) treatment. It was evident among all late cultivars and for two of the early cultivars; the exception was ‘Eliza’. Where present, this persistent component of the total vernalisation response delayed flowering in long days if the cultivars had experienced no VRN (e.g. King, a delay of 830 degree-days; Avila, 711 degree-days; Goulburn, 495 degree-days; Serratas, 448 degree-days; Izmir, 221 degree-days).

### Response to vernalisation under short and long days

The patterns of the responses by each cultivar to VRN treatment was further examined by graphing the thermal time to first flower when plants were grown under 8 h (Fig. 5a) and 20 h (Fig. 5b) PPD conditions.

The responses to VRN treatment under an 8 h PPD by all cultivars followed a similar pattern (Fig. 5a). The longest



**Fig. 5.** Thermal time to first flower appearance (degree-days) for early and late maturing yellow serradella ('King', 'Avila'), French serradella ('Eliza', 'Serratas') and subterranean clover cultivars ('Izmir', 'Goulburn') in response to non-inducing (a; 8 h PPD) and inducing (b; 20 h PPD) photoperiod and VRN treatments. The data points are back-transformed means of the  $\log_{10}$  data used for ANOVA. The error bars shown on each point are back-transformed l.s.d. bars based on the 3-way interaction l.s.d. ( $P = 0.05$ ). Where the bars for two means do not overlap, the means may be regarded as significantly different according to Fisher's 5% l.s.d. test. Arrows indicate approximate vernalisation thresholds representing  $V_{base}$  and  $V_{sat}$  for the cultivar 'King'.

time to first flower was observed without vernalisation. Time to flower was only reduced significantly after a period of VRN treatment had elapsed. The length of this period was cultivar specific, with early cultivars needing negligible (e.g. 'Eliza') or short (e.g. ~1 week, 'Izmir', 'King') VRN treatment periods, and late cultivars requiring longer periods (e.g.  $\geq 5$  weeks) in the VRN treatment. The response patterns of 'Eliza', 'Izmir', 'King' and 'Goulburn' indicated that each cultivar also exhibited a second VRN treatment threshold, when the time to flower had been reduced to a minimum value that represented their intrinsic earliness. However, this was not proven for 'Avila' or 'Serratas' because the longest VRN treatment period (9 weeks) used in the experiment was not sufficient to achieve their minimum time to flower in an 8 h photoperiod.

The duration of the VRN treatment required to eliminate delays to flowering under the 20 h PPD treatment (Fig. 5b) were substantially less than observed under the 8 h PPD

treatment for all cultivars (Fig. 5a). The VRN treatment thresholds also did not follow the same pattern. For example, the thermal time to flower by 'Serratas' under 8 h PPD was not reduced until after 7 weeks of VRN treatment, but the delay to flowering due to its persistent vernalisation requirement was eliminated within 1 week of the VRN treatment under the 20 h PPD regime.

## Discussion

### Measurement of time to first flower

In the present experiment, it was necessary to use a combination of node number and thermal time to first flower to fully investigate the flowering physiology of the legumes. Node of first flower is often used as an index of the occurrence of floral initiation, flowering time and, consequently, maturity type because the variability introduced by factors that affect plant growth rate (e.g. temperature, light intensity) can be reduced (Collins and Wilson 1974). This study and others (Aitken and Drake 1941; Evans 1959; Salisbury et al. 1987), demonstrated that this was a reliable index when first flowers appeared on a main or primary axis (e.g. plants grown with 16 and 20 h photoperiods). However, for plants grown in flowering physiology experiments, where treatments extend beyond the normal range of environmental stimuli (e.g. under an 8 h photoperiod and low VRN treatments, as in the present experiment), node number can become an unreliable indicator of flowering time among late maturing genotypes that begin to flower on secondary or higher order axes. This is most likely because rate of node development on higher order axes can occur at a slower rate than on the primary axis (Faverjón et al. 2017).

### Assumptions behind vernalisation response rankings

For plant genotypes with a vernalisation requirement, exposure to chilling temperatures hastens their time to flower. However, the effectiveness of chilling temperatures for vernalisation varies over a permissive range which is usually defined by three cardinal temperatures (minimum, optimum and maximum temperature for vernalisation; e.g. Penrose et al. 2003). Genotypes may differ in their cardinal temperatures but, where varieties-within-species have been studied, the differences are small. No difference in cardinal temperatures were found among three cultivars of wheat (*Triticum aestivum* L.) (i.e. minimum  $-3^{\circ}\text{C}$ , optimum  $6.5^{\circ}\text{C}$  and maximum  $15.9^{\circ}\text{C}$ ; Penrose et al. 2003) and only differences in the maximum effective temperature were observed among 15 lines of arabidopsis (*Arabidopsis thaliana* (L.) Heynh.) (Wollenberg and Amasino 2012).

Full specification of the cardinal temperatures for vernalisation is essential, for example, when attempting to predict

flowering under field conditions, where temperatures change diurnally and seasonally. However, a study of the full interaction of vernalisation temperatures and photoperiod responses would have been logistically complex and was, arguably, unnecessary for the objectives of the present experiment. Instead, we defined a constant temperature environment (5°C with an 8 h photoperiod) that should deliver a relative measure sufficient for ranking the cultivars by their response to vernalisation. A temperature of 5°C was considered likely to be very close to the optimum cardinal temperature for vernalisation for the genotypes in the present study. The evidence behind this decision was: (1) many experiments attest to the fact that subterranean clovers and serradellas are vernalised by exposure to temperatures within the range 3–7°C (Evans 1959; Gladstones and Devitt 1971; Aitken 1974; De Ruiter and Taylor 1979), (2) studies of other temperate plants indicate the optimum temperatures for vernalisation occur in the range 1–7°C, with many reports close to 5°C (e.g. 1°C (Brown *et al.* 2013) or 6.5°C (Penrose *et al.* 2003) for wheat, 5°C for chicory (*Cichorium intybus* L.) (Wiebe 1989, 1997) and leek (*Allium porrum* L.) (Wiebe 1994) and 4–7°C for arabidopsis (Wollenberg and Amasino 2012)).

The cultivars were ranked using the shortest time in the vernalisation treatment required to induce a reduction in the time to flower under short (8 h) days (Fig. 5a). The reliability of this relative measure of vernalisation requirement depends on it having been measured close to the optimum cardinal temperature for vernalisation where any differences, if they exist, in minimum and/or maximum cardinal temperatures would only have a relatively small impact on vernalisation effectiveness.

### Factors that determined maturity type

The maturity type of a pasture genotype is determined by its intrinsic earliness combined with the degree to which any vernalisation or photoperiod requirements are satisfied by the environment in which it is grown (Aitken 1974). The cultivars examined in this study were selected to represent very early and late maturity genotypes for southern Australian environments (Lattimore and McCormick 2012). Despite this, differences in the intrinsic earliness among genotypes within a species and among the legume species were relatively small (typically 900–1182 degree-days; Fig. 4) and did not explain the large differences in flowering time that are observed between the early and late maturing cultivars when grown in the natural environment (Boschma *et al.* 2019). Due to finite availability of controlled-environment space, only six genotypes could be studied in detail. Nevertheless, the minimum node of first flower observed for subterranean clover in the present study (Fig. 3), was consistent with that observed among a wider cohort of subterranean clover cultivars in previous studies (Aitken and Drake 1941; Aitken 1974). There were no equivalent data available for the serradellas.

The ranking of the early and late maturity phenotypes of the cultivars tested in the present experiment were inferred by the extent to which flowering was delayed when their requirements for vernalisation and long photoperiod stimuli were not met (i.e. when grown without vernalisation and under an 8 h photoperiod). Among the early cultivars, the longest time to first flower ranged from ~1912 ('Eliza') to ~2528 ('King' and 'Izmir') degree-days. In contrast, the longest time to first flower by the late cultivars ranged from 3515 ('Serratas') to 5244 ('Goulburn') degree-days (Fig. 4).

## The nature and interaction of vernalisation and photoperiod responses

### Vernalisation

The response to vernalisation under 8 h PPD was used to estimate the relative total vernalisation requirement of the cultivars. Our interpretation of vernalisation requirement using this measure assumes that an 8 h photoperiod does not induce a flowering response in the cultivars under study. This assumption may not be entirely safe because there are some long day, 'winter' cultivars of various species for which exposure to short days promotes floral initiation (e.g. wheat, Evans (1987); winter rye, Purvis and Gregory (1937); temperate grasses, Heide (1994)). However, we are not aware of any reports that annual pasture legumes are induced to flower under short days, short-day induction to flowering is not observed universally amongst long day plants, and the responses to short photoperiods observed in the present experiment were not similar to the form of short-day stimulation reported by Evans (1987). A detailed photoperiod study would be required to determine if separate 'primary' and 'secondary' induction requirements exist for floral initiation in the present cultivars (e.g. Heide 1990). Comparisons at 20 h were assumed to allow an estimate of the vernalisation required to eliminate the persistent component of vernalisation (i.e. that not substituted by the floral inducing effect of long photoperiods).

In the short photoperiod (Fig. 5a), the response of each cultivar to the vernalisation treatment was typically characterised by the need for a minimum period in the vernalisation treatment before the time to first flower was reduced below the cultivar's longest time to flower. This is analogous to the characteristic referred to as 'Vbase', as described in the winter wheat model ARCWHEAT1 (Weir *et al.* 1984), which has since been used in other studies of flowering in response to vernalisation (e.g. wheat, Gouache *et al.* 2017 and perennial ryegrass (*Lolium perenne* L.), Chynoweth 2021). The Vbase parameter is described in vernal day units and sets a requirement for a minimum (base) accrual of vernal days before the time to flower begins to decline. In most cases, it was possible to infer the maturity type ranking of the cultivars using the measure of Vbase (i.e. Eliza < Izmir = King < Avila ≈ Goulburn; Fig. 5a). However, cultivar 'Serratas' was an exception. It had a moderate delay to flowering in the

absence of vernalisation (ranking between 'Izmir'/'King' and 'Avila'/'Goulburn' in this respect), but the duration of VRN treatment needed to reduce time to flower was disproportionately greater than that anticipated from the responses of the other cultivars. There were insufficient data to explain why 'Serratas' appeared to differ from the other cultivars. However, it may be relevant that 'Serratas' was the only cultivar, of those tested, that was not developed from lines collected in the Mediterranean. It was collected from Rio Gallegos, Argentina at a very low latitude ( $-51.63$ ,  $-69.25$ ) in Patagonia (R Reid, pers. comm.; (APG 2022)).

Once 'Vbase' thresholds had been met, further exposure to the VRN treatment was needed to reduce the time to flower to a minimum value. For the cultivars where sufficient exposure to vernal temperature had been provided (i.e. 'Izmir', 'King', 'Eliza' and 'Goulburn'), this minimal value was equivalent to the intrinsic earliness of the cultivar because long VRN treatment times overrode any need to respond to photoperiod. The shortest duration in the VRN treatment to reach this second vernalisation threshold is analogous to the parameter referred to as 'Vsat' in other studies of flowering response to vernalisation (Weir *et al.* 1984; Brown *et al.* 2013; Chynoweth 2021). Vsat is one of the factors used to predict rate of progress to flowering. It is feasible that ranking the cultivars in the present study on the basis of Vsat would achieve a similar outcome to ranking according to Vbase, but the data in Fig. 5a were not strong enough to test this hypothesis because 9 weeks in the VRN treatment ( $5^{\circ}\text{C}$ ) was not long enough to saturate the vernalisation responses of two of the cultivars (i.e. 'Avila' and 'Serratas').

### Photoperiod

In the absence of a vernalisation stimulus (i.e. 0 VRN treatments, Figs 3, 4), all the cultivars expressed their longest time to flower in short photoperiods up to a threshold photoperiod above which the time to flower was reduced. This threshold is effectively analogous to the characteristic referred to as Pbase (Weir *et al.* 1984; Roberts and Summerfield 1987). For the late maturing cultivars this occurred at photoperiods between 8 and 12 h, but it may have been  $\leq 8$  h among the early cultivars (Fig. 4).

Among the late cultivars, in particular, a precipitous decline in the need for vernalisation to reduce time to flower, occurred as photoperiod was increased from 12 to 16 h. This was revealed by large and rapid reductions in the thermal time to flower and in the node of first flower. Although we have linked these data (Figs 3 and 4) by assuming linearity between PPD 12 and PPD 16, the precipitous decline in time to flower may well be better described by a reverse sigmoidal response with the time to flower becoming unresponsive to lengthening photoperiods in photoperiods somewhere between 12 and 16 h. This photoperiod threshold is analogous to Psat (Weir *et al.* 1984) and is also described as a 'critical photoperiod' (Roberts and Summerfield 1987; Evans *et al.* 1992). For 'long day' plants, critical photoperiod defines the

photoperiod above which progress to flowering is only sensitive to temperature.

Critical photoperiods for flowering have been estimated in several 'short day' and 'long day' legume species by measuring time to flower under constant photoperiods set at hourly increments between 11.5 and 14.5 h photoperiod (Keatinge *et al.* 1998), but neither subterranean clover nor the serradella species have been included in such studies. However, data from previous experiments with subterranean clover do indicate that there is capacity for long photoperiods to reduce requirements for vernalisation and *vice versa* (Aitken 1955). Aitken (1955) also reported a precipitous decline in node number to first flower between 12 and 15 h photoperiod treatments using unvernalsed plants of the early maturing subterranean clover cultivar 'Dwalganup'. More frequently, critical photoperiods are estimated based on flowering time responses of cultivars from monthly sowings under natural photoperiod and temperature regimes. Average photoperiods and temperatures experienced between sowing and flowering are used to estimate critical photoperiods using linear functions for rate of progress in time to flower (Roberts and Summerfield 1987; Evans *et al.* 1992). We consider that the 4 h photoperiod interval used in the present experiment was too wide to allow a critical photoperiod to be defined with any accuracy, or to even determine whether it was similar for all, or some of the cultivars. This will need to be the subject of future experiments using narrower photoperiod intervals.

### Vernalisation $\times$ photoperiod interactions

The implications of photoperiod overriding the need for vernalisation are substantial. For large areas of temperate southern Australia (e.g. at latitudes  $30$ – $50^{\circ}\text{S}$ ), many cultivars will germinate and grow in photoperiods unlikely to induce flowering ( $<12$  h) between mid-April and late August. Under these conditions, vernalisation will initially be the dominant influence on the timing of flowering. However, the present experiment also demonstrated that, for many of the cultivars, responses to photoperiod and vernalisation are highly interactive. As these legumes grow into lengthening photoperiods (i.e. after winter), their need for vernalisation will be greatly reduced. Lengthening days and a rapid thermal time step during spring effectively eliminate any further delay to flowering that might otherwise be expected if vernalisation requirements were not met during winter. Pbase and the critical photoperiod are, consequently, among the defining characteristics of a cultivar's maturity type and flowering date stability.

### Persistent vernalisation requirement

Despite the strong overriding influence of a long photoperiod on the need for vernalisation, photoperiod did not completely override the requirement for some vernalisation in five of the six cultivars. A persistent requirement for some vernalisation was observed even under a 20 h photoperiod when the cultivars had experienced only mild or non-vernalising

temperatures (Figs 4, 5b). Only 'Eliza' did not exhibit a persistent vernalisation requirement. The existence of a vernalisation requirement that is not eliminated by long photoperiods can also be seen in the node of first flower data for subterranean clover presented by Aitken (1955) and Evans (1959), although they did not highlight the importance of a persistent vernalisation requirement. A genetic basis for a persistent vernalisation requirement in pasture legumes has been revealed by genetic analysis of barrel medic (*Medicago truncatula* Gaertn.). The presence of the *MtVRN2* gene slows the transition to flowering under long days in the absence of vernalisation (Jaudal *et al.* 2016).

We surmise that a persistent requirement for some vernalisation may help a cultivar avoid very premature flowering in years when the autumn break of season occurs in late summer or early autumn under long days and warm conditions. For example, full expression of the persistent vernalisation requirement under the conditions of the present experiment substantially extended the flowering time of cultivar 'King' from 923 degree-days (i.e. intrinsic earliness) to 1753 degree-days (zero vernalisation, long photoperiods  $\geq 16$  h; Figs 4, 5b). If 'King' were to germinate on early rain in early autumn (mid-March) in Coonabarabran (NSW) where this cultivar is used, a flowering time delay of this magnitude is likely to ensure that flowering would not be induced before the plant was experiencing the cool conditions of winter under short photoperiods.

### Flowering date stability

Some serradella cultivars are reported to have poor flowering date stability when grown in key locations in southern Australia: i.e. their flowering dates were earlier after an early germination event (Boschma *et al.* 2019; Haling *et al.* 2023). The current data reveal factors that are likely to be important for achievement of stable flowering dates:

- (1) A delay to flowering caused by a persistent component of the total vernalisation requirement.

In temperate and Mediterranean areas of southern Australia, autumn germination events are likely to occur under warm temperatures within a photoperiod range of 11–13 h, (i.e. typically centred around mid-autumn [April]). A persistent component of vernalisation requirement that is not eliminated by long days, effectively helps to prevent early floral initiation after very early germination events (late summer/early autumn) during long photoperiods (i.e.  $\geq 13$  h). Under these conditions a persistent vernalisation requirement can potentially delay flower induction until the onset of cooler winter temperatures. The existence of a cultivar that lacked a persistent vernalisation requirement (i.e. 'Eliza'; Figs 3 and 4) and evidence of a genetic basis (Jaudal *et al.* 2016) for this attribute indicates it is potentially manipulable during plant breeding.

- (2) The magnitude of the potential delay to flowering due to a plant's vernalisation requirement (i.e. as measured in the present experiment under short photoperiods).

As autumn transitions to winter, plants are growing in relatively short photoperiods (<11 h) and the coolest temperatures of the year. At this time of year, vernalisation is the dominant factor (other than thermal time accrual) modifying a cultivar's time to flower. To favour stable flowering in an environment with highly variable germination dates, pasture legumes require a vernal time requirement for *Vbase* that is (at least) greater than the potential for vernal time accrued during the period over which germination events may occur. Irrespective of this, *Vbase* may not be sufficient to ensure a stable flowering date in all circumstances. For example, long photoperiods that coincide with very early germination events may erode a large component of the delay to flowering that is associated with a cultivar's need for vernalisation. In this instance, it may be essential that a cultivar also exhibit a persistent requirement for some vernalisation to protect against premature flowering.

- (3) The interaction of photoperiod and vernalisation responses.

Seasonal temperature conditions are often characteristic of a location, but they also vary interannually and plants requiring vernalisation to progress more rapidly to flowering may not be vernalised to the same extent in every growing season. However, as photoperiod increases, the response to photoperiod overrides the 'non-persistent component' of a cultivar's need for vernalisation to hasten flowering. This assists the plant to progress rapidly to flowering irrespective of variations in the winter temperature regime. The culmination of this interaction of responses to vernalisation and photoperiod is a critical photoperiod above which there is no further reduction in time to flower due to lengthening days. Flowering time is, thereafter, only modulated by temperature. In southern Australia, lengthening days after the winter solstice typically mean that a 12 h photoperiod (inclusive of civil twilight) is reached by the end of August (i.e. in late winter). Even cultivars with a considerable vernalisation requirement (e.g. late maturing types) would have their vernal temperature requirement diminished or cancelled by early spring as a result of the overriding influence of photoperiod.

### Conclusion

At the commencement of this study, we hypothesised that responses to both vernalisation and photoperiod (long days) would be important determinants of maturity type among cultivars of yellow and French serradellas. The experiment has shown that differences among cultivars in their intrinsic earliness were not large enough to explain a cultivar's

maturity type. Instead, maturity type was associated with the magnitude of the potential delays to flowering that occurred when a cultivar's requirements for vernalisation and photoperiod stimuli were not met. Time to flower among early maturing cultivars was delayed by relatively short periods; longer flowering time delays occurred for late maturing cultivars. Later maturing cultivars required longer exposure to vernalising temperatures than early cultivars before time to flower was reduced. However, if their need for vernalisation were not met fully during autumn–winter, lengthening photoperiods in spring would override any remaining vernalisation requirement to limit further delay to flowering. Maturity type of the serradella cultivars was thus determined in a similar way to maturity type among the subterranean clover cultivars.

The results lead us to surmise that stable flowering dates are more likely if a cultivar displays: (1) a component of its vernalisation requirement that is not overridden by long photoperiods to protect against premature flowering if seeds germinate under long, warm photoperiods in late summer–early autumn, (2) sufficient requirement for exposure to vernalising temperatures to ensure the transition to flowering occurs after autumn–winter irrespective of germination date, and (3) a response to lengthening photoperiods that overrides any vernalisation requirement not met fully by the end of winter.

## Supplementary material

Supplementary material is available [online](#).

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**Data availability.** The data that support this study will be shared upon reasonable request to the corresponding author.

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