

1 **Interactive effects of elevated CO₂ and drought on nocturnal water fluxes in**
2 ***Eucalyptus saligna***

3
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23
24 Running title: CO₂ and drought affect nocturnal sap flow

25

1 **Summary**

2

3 Nocturnal water flux has been observed in trees under a variety of environmental conditions and
4 can be a significant contributor to diel canopy water flux. Elevated atmospheric CO₂ (elevated
5 [CO₂]) can have an important effect on day-time plant water fluxes, but it is not known whether it
6 also affects nocturnal water fluxes. We examined the effects of elevated [CO₂] on nocturnal water
7 flux of field-grown *Eucalyptus saligna* trees using sap flux through the tree stem expressed on a
8 sapwood area (J_s) and leaf area (E_t) basis. After 19 months growth under well watered conditions,
9 drought was imposed by withholding water for five months in the summer, ending with a rain event
10 that restored soil moisture. Reductions in J_s and E_t were observed during the severe drought period
11 in the dry treatment under elevated [CO₂], but not during moderate- and post-drought periods.
12 Elevated [CO₂] affected night-time sap flux density which included the stem recharge period, called
13 ‘total night flux’ (19:00 to 05:00, $J_{s,r}$), but not during the post-recharge period, which primarily
14 consisted of canopy transpiration (23:00 to 05:00, $J_{s,c}$). Elevated [CO₂] wet (EW) trees exhibited
15 higher $J_{s,r}$ than ambient [CO₂] wet trees (AW) indicating greater water flux in elevated [CO₂] under
16 well-watered conditions. However, under drought conditions, elevated [CO₂] dry (ED) trees
17 exhibited significantly lower $J_{s,r}$ than ambient [CO₂] dry trees (AD) indicating less water flux
18 during stem recharge under elevated [CO₂]. $J_{s,c}$ did not differ between ambient and elevated [CO₂].
19 Vapour pressure deficit (D), was clearly the major influence on night-time sap flux. D was
20 positively correlated with $J_{s,r}$ and had its greatest impact on $J_{s,r}$ at high D in ambient [CO₂]. Our
21 results suggest that elevated [CO₂] may reduce night-time water flux in *E. saligna* when soil water
22 content (SWC) is low and D is high. While elevated [CO₂] affected $J_{s,r}$, it did not affect day-time
23 water flux in wet soil, suggesting that the responses of $J_{s,r}$ to environmental factors cannot be
24 directly inferred from day-time patterns. Changes in $J_{s,r}$ are likely to influence pre-dawn leaf water
25 potential, and plant responses to water stress. Nocturnal fluxes are clearly important for predicting
26 effects of climate change on forest physiology and hydrology.

1

2 **Abbreviations:**

3

4 D = vapour pressure deficit (kPa); E_n = nocturnal sap flow; E_t = transpiration per leaf area ($\text{mm}^3 \text{hr}^{-1} \text{m}^{-2}$ leaf area); $E_{t,r}$ transpiration per leaf area including recharge component or ‘total night
5 transpiration’ (19:00 to 05:00); $E_{t,c}$ transpiration per leaf area with canopy transpiration only or
6 ‘post-recharge transpiration’ (23:00 to 05:00); g_n = nocturnal leaf conductance (sum of stomatal
7 and cuticular conductance, $\text{mmol m}^{-2} \text{s}^{-1}$); g_d = day-time stomatal conductance, ($\text{mmol m}^{-2} \text{s}^{-1}$); J_s -
8 sap flux density; $J_{s,r}$ = nocturnal sap flux density including recharge component ‘total night flux’
9 (19:00 to 05:00); $J_{s,c}$ = post-recharge flux density (23:00 to 05:00); Ψ_{pd} = pre-dawn leaf water
10 potential; *WTC* = Whole Tree Chambers. Treatments as follows: AD = Ambient [CO_2] and dry soil;
11 ED = elevated [CO_2] and dry soil; AW = ambient [CO_2] and wet soil; EW = elevated [CO_2] and
12 wet soil.
13

14

15

1 **Introduction**

2

3 Nocturnal water flux is a wide-spread phenomenon that has been observed in a variety of
4 ecosystems and plant functional groups (Caird et al. 2007; Dawson et al. 2007; Snyder et al. 2003),
5 including trees (Daley and Phillips 2006; Fisher et al. 2007). Water flux at night may occur as sap
6 flux (E_n) through the tree stem and as water loss through the leaf via stomatal and cuticular
7 conductance (g_n). While it has been suggested that nocturnal water flux is primarily a function of
8 stem refilling (*i.e.* recharge) (Caird et al. 2007), most studies indicate that water loss through the
9 leaves is >50% of nocturnal sap flow (Caird et al. 2007; Zeppel et al. 2010). In turn, stomata
10 account for the major proportion, and cuticular conductance for <10% of total leaf conductance at
11 night (Phillips et al. 2010; Zeppel et al. 2010).

12 Nocturnal water flux can be a sizable component of whole-plant water use. In some arid
13 ecosystems, E_n may reach 30-60% of day-time water flux (Snyder et al. 2003), but in most
14 ecosystems it is rare for E_n to exceed 20% of day-time flux (Dawson et al. 2007). In *Eucalyptus*, E_n
15 consistently averaged 5-8% of day-time flux across all seasons, occasionally reaching 20% of day-
16 time flux on a single day (Phillips et al. 2010; Zeppel et al. 2010). E_n in eucalypts is strongly and
17 positively correlated with higher vapour pressure deficit (D), such that water flux is largest on high
18 temperature, low humidity nights (Phillips et al. 2010; Zeppel et al. 2010), as has been frequently
19 observed in other species (Daley and Phillips 2006). E_n in eucalypts is also higher in wetter soils,
20 but soil moisture is generally a weak predictor of E_n compared with D (Zeppel et al. 2010).

21

22 While the influence of D on nocturnal fluxes is well described, the effects of elevated CO_2 on
23 nocturnal fluxes have not been studied. Trees commonly show reduced leaf-level stomatal
24 conductance during the day (g_d) in elevated $[\text{CO}_2]$, due to partial stomatal closure, which reduces
25 leaf-level water flux (Ainsworth and Rogers 2007; Medlyn et al. 2001). This change in leaf-level
26 stomatal conductance can have significant consequences for whole-plant water use, soil moisture

1 content and the impacts of drought stress (Field et al. 1995; Wullschleger et al. 2002). Droughts are
2 widely predicted to increase in frequency and severity (IPCC, 2007) in concert with rising [CO₂]
3 and there is a clear and obvious need to study their interactive effects on a range of plant water
4 relations. At the whole tree level, lower g_d may be partially or completely offset by larger leaf area
5 production in elevated [CO₂]. Therefore, the net effect of elevated [CO₂] on sap flux on a sapwood-
6 area basis (J_s) reflects combined effects of g_d and whole canopy leaf area (Cech et al. 2003;
7 Ainsworth et al. 2005; Uddling et al. 2008). However, the effects of [CO₂] on nocturnal fluxes are
8 not yet known, but have the potential to either strengthen, or counteract, effects on water balance
9 through changes in day-time water flux, and therefore need to be quantified. In addition, both [CO₂]
10 and nocturnal fluxes influence the level of drought stress that a plant experiences (Howard et al.
11 2009).

12
13 While it is not known how rising [CO₂] affects nocturnal water flux, there is some evidence that
14 day-time and nocturnal water fluxes are correlated (Marks and Lechowicz 2007). Therefore, an
15 initial hypothesis for the effect of elevated [CO₂] on nocturnal fluxes would be that it would mimic
16 the effect on day-time water flux; that is, if daytime transpiration rate was reduced under elevated
17 [CO₂] then night-time transpiration rate would also be reduced. However, there are key differences
18 in the regulation of nocturnal and day-time water fluxes.

19
20 Day-time water flux is at least partially under active *biological* control through opening and closing
21 of stomata. The reduction in stomatal conductance to elevated [CO₂] reflects biological control
22 balancing carbon uptake with water loss (Medlyn et al. 2010). In contrast, nocturnal water flux is
23 primarily considered (at present) to be under *physical* control, particularly by D and soil moisture
24 (Dawson et al. 2007; Phillips et al. 2010; Zeppel et al. 2010). If night-time sap flux is not under
25 active *biological* control, it may be hypothesised that there would be no direct response to rising
26 atmospheric [CO₂]. Hence, the major effect of rising [CO₂] on night-time water flux might be via

1 direct responses to changes in soil water content which result from day-time sap flux. Lower day-
2 time sap flux under high [CO₂] might lead to improved stem water status, and therefore a reduced
3 need to refill xylem, resulting in lower night time sap flux. Consequently, we hypothesised that
4 elevated [CO₂] effects on night-time sap flux would follow changes in day-time sap flux.

5
6 This study is the first to address the interactive effects of elevated [CO₂] and drought on nocturnal
7 water flux. We studied *Eucalyptus saligna* Sm. saplings grown in closed-top, environmentally
8 controlled whole-tree chambers in the field (Barton et al. 2010). Trees were exposed to either
9 ambient or elevated [CO₂] for a total of 24 months. In the final five months of the experiment,
10 water was withheld from half of the experimental trees, resulting in four experimental treatments:
11 (1) ambient CO₂, wet soils (AW); (2) ambient CO₂, dry soils (AD); (3) elevated CO₂, wet soils
12 (EW); and (4) elevated CO₂, dry soils (ED) under ambient field temperature and light conditions. In
13 the dry soil treatments, water was withheld starting in October and ending with a rain event in
14 February.

15
16 In this experiment, day-time transpiration was found to be strongly affected by elevated [CO₂]
17 treatment. The dry soil treatments reduced day-time transpiration to a greater degree under elevated
18 [CO₂] than under ambient [CO₂] due to shallower rooting depth at elevated [CO₂], ((Duursma et al.
19 2011). Here, we report the effects of elevated [CO₂] on night-time sap fluxes of saplings grown in
20 wet or dry soils. We assessed the impact of the [CO₂] and drought treatments during the night on J_s
21 and E_t as a function of volumetric soil water content (SWC), vapour pressure deficit (D), and pre-
22 dawn leaf water potential (Ψ_{pd}). Our goal was to assess whether J_s and E_t would be affected by
23 elevated [CO₂] or severe summer drought, and whether night-time fluxes follow day-time patterns
24 in response to elevated [CO₂].

1 Specifically, we hypothesised that (1) elevated $[\text{CO}_2]$ would decrease J_s and E_t ; (2) drought would
2 reduce J_s and E_t ; and (3) elevated $[\text{CO}_2]$ trees would be less drought stressed and therefore the
3 reduction in J_s and E_t due to drought would be less under elevated $[\text{CO}_2]$.

4

5 **Materials and methods**

6 *Study site*

7 Research was conducted at the Hawkesbury Forest Experiment (HFE) site in western Sydney
8 (Australia) (33°36'40" S, 150°44'26.5" E) at elevation 25 m. a.s.l. Site details are described in
9 (Barton et al. 2010). The local climate is considered to be warm and temperate, with long-term
10 mean annual rainfall at the nearest weather station (Richmond) of ~800 mm (1900 to 1990). Over
11 the measurement period (December 2008 to March 2009) mean temperature was 22.7 °C with
12 maximum temperature of 43.7 °C and minimum temperature of 7.8 °C; precipitation was 254 mm.
13 Trees were exposed to CO_2 treatments from April 2007 to March 2009. This experiment focuses on
14 the drought period during summer December 2008 to March 2009. At the start of this period, the
15 trees were approximately 6 to 9 m tall with a diameter of 75 to 125 mm at approximately 1.10 m
16 height.

17

18 *Experimental Design*

19 Twelve whole-tree chambers (WTCs) were used to control atmospheric CO_2 throughout the
20 experimental period; these are fully described in Barton et al. (2010). Briefly, six WTCs were set to
21 track ambient CO_2 and six chambers were operated at elevated CO_2 (ambient + 240 ppm). All
22 WTCs were maintained at ambient outside temperature. Within each of the $[\text{CO}_2]$ treatments, three
23 WTCs were irrigated every 3 days with 10 mm of water; due to hot weather, irrigation was
24 increased to 12 mm every three days from 15th January 2009 onward. In the remaining three WTCs
25 in each CO_2 treatment, trees were irrigated as described above until the drought treatment was

1 initiated in October 2008 by a dry-down in which water was completely withheld. Trees were
2 grown under the four [CO₂] x drought treatments under ambient field temperature and light
3 conditions for five months before drought trees were re-watered. The post-drought period began
4 when heavy rains ended the drought in mid-February.

5

6 *Soil Moisture*

7 The impact of the irrigation and drought treatments on soil moisture was assessed using Sentek
8 probes (Campbell Pacific Nuclear International (CPN) Model 503) inserted into an access tube,
9 located approximately 0.8 m distance from the base of the tree, and measured at soil depths of 0.25,
10 0.5, 0.75 and 1.0 m within each whole tree chamber plot. Data were calibrated using field
11 techniques outlined by (Greacen et al. 1981). Soil moisture data were corrected by calibrating data
12 to 'field capacity' during the days when flooding occurred (Duursma et al. 2011).

13

14 *Leaf Water Potential*

15 Bulk leaf water potential was measured at pre-dawn (Ψ_{pd} , MPa) each month on two fully expanded
16 leaves per tree in each whole tree chamber and control plot. Leaves were removed from the middle-
17 to upper-canopy and immediately sealed in a foil-laminate bag. Measurements of Ψ_{pd} were
18 conducted using a Scholander-style pressure chamber (Model 600, PMS Instrument Company,
19 Corvallis, OR, USA).

20

21 *Sap flux density*

22 Sap velocity was measured using the heat ratio method (HRM) as described by Burgess et al.,
23 (2001). One probe set (with 2 sensors per tree) was inserted into each tree at *ca.* 1.3 m height. The
24 radial profile of sap velocity was examined in two nearby control trees by inserting the probe set
25 deeply into the sapwood, and then withdrawing the unit 5mm at 30-minute intervals over the entire

1 width of the sapwood; these data were compared with data from a probe set which remained in a
2 single location (Ford et al. 2004; Medhurst et al. 2002). Sap velocity was measured at 15-minute
3 intervals throughout the experimental period (December 2008 to March 2009). Heat Ratio data
4 were converted to sap flux density (J_s , $\text{mm}^3 \text{ water mm}^{-2} \text{ sapwood area hr}^{-1}$) by correcting for the
5 effects of wounding, radial variability in flow, sapwood area and volumetric fractions of water and
6 wood using algorithms described by Burgess et al. (2001). Wound width may affect calculation of
7 whole tree water use, so a different wound width was determined for each tree; wound widths
8 varied from 1.60 ± 0.02 to 1.95 ± 0.10 mm (Zeppel et al. 2008a). Wood moisture content ((fresh
9 weight – dry weight)/dry weight) and density also affect sap flow calculations; however, there were
10 no significant differences across treatments ($p > 0.05$). Variation in moisture content of sapwood
11 across trees ranged from 63 to 66 % (s.e. < 1.5 %). Mean (and s.e.) wood density was 4.1 ± 0.4 and
12 $4.0 \pm 0.3 \text{ mm g}^{-1}$ for ED and AD treatments, respectively.

13
14 HRM data are accurate when sap flow values are low; however, they potentially under-predict sap
15 flow during periods of very high water flux ($> 400 \text{ mm hr}^{-1}$) (Madurapperuma et al. 2009) which
16 may occur in *E. saligna* during the day-time. Therefore, sap flow at night was determined using
17 HRM probes and day-time sap flow was calculated from whole tree chamber water flux
18 measurements (Barton et al 2010); HRM probes were calibrated against whole tree chamber data.

19
20 In order to account for differences in tree size, we normalised water flux on a sapwood area basis
21 (*i.e.* sap flux density; J_s , $\text{mm}^3 \text{ water mm}^{-2} \text{ sapwood area hr}^{-1}$) and leaf area basis (*i.e.* transpiration;
22 E_t , $\text{mm}^3 \text{ water m}^{-2} \text{ leaf area hr}^{-1}$). We calculated two night-time sap flow terms:

23 (1) Total night flux, which is calculated as the total sap flow over the period when solar radiation
24 was zero, *ca.* 19:00 until 05:00. This term includes stem water recharge and canopy transpiration
25 and is denoted $J_{s,r}$ and (2) post-recharge flux, which is calculated as the sap flow over the period
26 from 23:00 to 05:00. This term reflects canopy transpiration only following Zeppel et al. (2010)

1 and is denoted $J_{s,c}$. These two definitions allowed an assessment of the impact of our [CO₂] and
2 drought treatments on two different aspects of nocturnal water use.

3

4 *Zero-set of sap flow*

5 Accurate estimation of the zero-set of each sensor is important when using many types of heat-
6 based sap flow systems (Regalado and Ritter 2007), including the HRM. A sensitivity analysis was
7 conducted on the effect of changing the zero offset on daily sap flow. When the zero offset was
8 increased by 10%, 20% and 50%, the hourly sap flux was decreased by 8%, 9% and 23%,
9 respectively. In this study, the zero-set of each sensor was determined using a modification of the
10 technique of Ward et al. (2008), by using periods when whole tree chamber water fluxes were zero
11 (between 24:00 and 05:00), and then within these periods, estimating the flow rates when D was
12 zero (Ward et al. 2008).

13

14 *Statistical analyses*

15 Data were tested for normality and the homogeneity of variances by plotting residuals versus
16 predicted values. These tests indicated the assumptions of normality and homogeneity of variances
17 were met for all variables and no transformations were necessary. Statistically significant
18 differences in soil water potential within the dry treatments, compared with the wet treatments,
19 became apparent from January 10 onward and were used to designate three distinct periods in this
20 study: ‘moderate-drought’ (5 December to 9 January 2009), ‘severe-drought’ (January 10 to
21 February 14 2009), and ‘post-drought’ (February 15 to March 14 2009; Table 1), and then trees
22 were harvested in mid-March 2009. Moderate and severe drought occur within the period defined
23 as ‘late drought’ in Duursma et al. (*this issue*). We used a repeated measures, two-way analysis of
24 variance to examine the main and interactive effects of CO₂ and water treatment on J_s and E_t . $J_{s,r}$ and

1 $J_{s,c}$ (between 19:00 to 05:00 and 23:00 to 05:00, respectively) and $E_{t,r}$ and $E_{t,c}$ (between 19:00 to
2 05:00 and 23:00 to 05:00, respectively) were dependent variables, and $[CO_2]$ and water treatment
3 were fixed, categorical factors. Statistical significance was determined as $P \leq 0.05$. Second order
4 polynomial regressions were used to assess the relationship between sap flux density and D . Mean
5 and error bars of ratios in Fig. 3 are determined following previously described methods (Lewis et
6 al. 2010). All statistical analyses were conducted using R version 2.11.1 (R Development Core
7 Team, 2010).

8

9 **Results**

10 *Environmental conditions*

11 Temperature at night was similar across the moderate-, severe- and post-drought periods, and
12 ranged from a minimum of 8 °C to a maximum of 25 °C (Fig. 1 a). D averaged within the two
13 night-time periods ranged from 0.05 to 0.9 kPa (Fig. 1 b). Minimal precipitation was received
14 outside the chambers from December 15 until February 14; afterward, heavy rainfall occurred over
15 a 10-day period (Fig 1c). During the severe drought period, wet treatment chambers had
16 consistently higher volumetric soil water content at 0.30 and 0.70 m depths, and over the total soil
17 volume, but not at 1.10 or 1.50 m depths, compared with the dry chambers during the severe
18 drought period (Fig. 1 d and e; Table 1). Soil water content gradually declined in both wet and dry
19 chambers during the peak, hot summer period until heavy rains ended the drought in mid-February.

20

21 *Pre-dawn leaf water potential*

22

23 Water limitation was imposed by ceasing irrigation inside the whole tree chambers in the dry
24 treatments beginning in October 2008. $[CO_2]$ had no significant effect on pre-dawn leaf water
25 potential, whereas water treatment had a significant effect ($p < 0.05$) and there was an indication of

1 an interaction with [CO₂] and water treatment during the severe drought (p = 0.055) (Fig. 1f; Table
2 2). As the drought progressed the ψ_{pd} in the dry treatments declined to lower values than in the
3 watered treatments in both [CO₂] treatments. There were no [CO₂] treatment effects on either the
4 moderate-drought or post-drought ψ_{pd} .

5

6 *Effects of tree size on sap flux density at night*

7

8 Tree size may affect tree water use, sap flux density (J_s) and transpiration (E_t) independently of
9 [CO₂] or drought treatments (Zeppel and Eamus 2008). Therefore, we examined whether
10 differences in water fluxes could be attributed to differences in tree size, specifically total leaf area
11 and tree sapwood area. There was no significant relationship between J_s and either leaf area or
12 sapwood area for the [CO₂] and water treatments (P>0.10). Similarly, there was no significant
13 relationship for E_t and either leaf area or sapwood area for any treatment.

14

15 *Interactive effects of elevated [CO₂] and water treatment on nocturnal sap flux density*

16

17 *Total night flux*

18 The effect of elevated [CO₂] on total night flux ($J_{s,r}$ and $E_{t,r}$) differed between wet and dry
19 treatments. In the wet treatments, elevated [CO₂] caused total night flux (per leaf area) to
20 significantly increase by 28% (p<0.05). However in the dry treatments, elevated [CO₂] caused total
21 night flux (per leaf area) to decrease by 33% (Fig. 2c-d; Table 2). Total night sap flux density (per
22 sapwood area) $J_{s,r}$ showed the same trends as transpiration per leaf area.

23

24 *Post-recharge flux*

25 Results were slightly different when only the post-recharge was considered. As for total night flux
26 per unit sapwood area, there was a significant decrease in post-recharge night flux under elevated

1 [CO₂] in the dry treatments. However, in the wet treatments, there was no significant difference
2 between [CO₂] treatments (Fig. 2e-h; Table 2). On a sapwood area basis, results were slightly
3 different again. There was a significant [CO₂] x drought interaction under elevated [CO₂], post-
4 recharge sap flux density was lower in the dry treatment than in the wet treatment [CO₂] (Fig. 2f),
5 but no such drought effect was observed under wet and dry ambient [CO₂] treatments (Fig. 2e).
6 There were no other significant differences among treatments for $J_{s,c}$. On a leaf area basis for $E_{t,c}$,
7 AD was significantly higher than ED (Table 2).

8

9 *Comparison of day vs night water fluxes*

10

11 As a result of the differential treatment effects of elevated [CO₂] on $J_{s,r}$ in the dry and wet
12 treatments during recharge, elevated [CO₂] was associated with a 33% *reduction* in $J_{s,r}$ in the dry
13 treatment but with a 28% *increase* in the wet treatment (Fig 3b); similar patterns were observed in
14 $E_{t,r}$ (Fig 3e). Elevated [CO₂] did not significantly affect $J_{s,c}$ or $E_{t,c}$ in either watering treatment (Fig
15 3c and f). During the day-time, elevated [CO₂] did not strongly affect E_t in the wet treatment,
16 however there was a reduction in the dry treatment (Fig 3a, d). $J_{s,r}$, $J_{s,c}$ and E_t summed over the
17 drought period were not significantly different among the four treatments (Figure 4). However, $J_{s,r}$
18 was significantly lower in ED compared with AD, whereas there was no significant difference
19 between $J_{s,r}$ of AW and EW ($P < 0.05$).

20

21 *Environmental regulation of sap flux density*

22

23 Both soil moisture content and D exert strong control over night-time fluxes (Fig. 5, 6). There were
24 positive correlations between night-time fluxes and D but those relationships differed with [CO₂]
25 and soil moisture availability. In wet soil, total night transpiration was higher at elevated [CO₂]
26 compared with ambient [CO₂] at a given D (Fig. 5c). In contrast, in dry treatments, total night

1 transpiration and post-recharge flux were higher at ambient [CO₂] compared with elevated [CO₂]
2 for any given D , particularly when $D > 0.25$ kPa (Fig. 5a,b). Windspeed (U) inside each chamber
3 was high, with visibly fluttering leaves, thereby reducing the leaf boundary layer, and comparable
4 across all treatments (Barton, unpublished data.). Therefore, the relationship between E_n and U , and
5 the product of U and D were not analysed (see Phillips et al. 2010 and Zeppel et al. 2010).

6
7 As drought progressed and soil moisture decreased, $J_{s,r}$ decreased in both AD and ED treatments
8 (Fig. 6). For a given D , $J_{s,r}$ was higher in the moderate drought period compared with the severe
9 drought period in both AD (Fig 6a) and ED (Fig 6b) treatments. Towards the end of the drought,
10 and when D was not high, nocturnal sap flux was occasionally negative. Negative flow rates have
11 been previously reported under dry conditions using the Heat Ratio Method which is accurate under
12 low flow rates (Burgess et al. 2001; Burgess et al. 1998). Soil moisture thus has an important effect
13 on night time fluxes, in that sapflux at a given D is reduced as soil moisture availability declines.
14 The physical control of night time fluxes increased as soil moisture declined, as demonstrated by a
15 much stronger correlation between sapflux and D in dry soil treatments than in well-watered
16 treatments (Fig. 5).

17

18 **Discussion**

19 This study was the first to quantify the effect of elevated [CO₂] on nocturnal water fluxes. We
20 hypothesised that: (1) elevated [CO₂] would decrease J_s and E_t , which was confirmed for dry soil
21 but not for wet soil; (2) drought would reduce J_s and E_t , which was confirmed; and (3) elevated
22 [CO₂] trees would be less drought stressed and therefore the reduction in J_s and E_t due to drought
23 would be less under elevated [CO₂], which was rejected. Elevated [CO₂] significantly reduced $J_{s,r}$
24 in trees in the dry treatment, but increased $J_{s,r}$ in trees in the wet treatment, compared with plants

1 grown in ambient [CO₂]. In contrast, elevated [CO₂] did not alter $J_{s,c}$ or water fluxes for wet
2 treatment plants during the day-time, compared with wet treatment plants grown in ambient [CO₂].
3 The primary driver of $J_{s,r}$ was D , which was positively correlated with $J_{s,r}$ and had a proportionately
4 greater impact on $J_{s,r}$ at high D in AD trees compared to ED trees. This study suggests that elevated
5 [CO₂] may reduce $J_{s,r}$ in *E. saligna* when soil water content is low and D is high, which often
6 occurs during temperate summers (Zeppel et al. 2008b).

7
8 Our results found contradictory changes in water fluxes under elevated [CO₂] with *increased*
9 transpiration per leaf area $E_{t,r}$ under elevated [CO₂] in the wet treatment, but *decreased* $E_{t,r}$ in the
10 dry treatment. In previous studies conducted during the day, [CO₂] and drought interactions have
11 been observed. For example, in a Free Air CO₂ Enrichment (FACE) experiment, *P. taeda*
12 transpired less water per unit of leaf area only when drought was severe (Schäfer et al. 2002).
13 *Liquidambar styraciflua*, the other major species in the forest, used progressively less water,
14 resulting in a 25% reduction in sap flux density after 3.5 years under elevated [CO₂] (Schäfer et al.
15 2002). In contrast, cherry and peach seedlings grown in environmentally controlled conditions did
16 not exhibit a [CO₂] x drought interaction, partly because increased leaf area under elevated [CO₂]
17 compensated for lower water use per leaf area (Centritto et al. 1999). Similarly, other [CO₂] studies
18 have failed to provide evidence that the effect of elevated [CO₂] on sap flow is enhanced during dry
19 conditions (Bobich et al. 2010; Hymus et al. 2003).

20
21 Although reductions in sap flow under elevated [CO₂] during the day have been observed in trees
22 (Schäfer et al. 2002), experimental evidence remains equivocal. For example, after an initial
23 increase in annual day-time sap flow of 14% in the first year of elevated [CO₂] treatment, annual
24 sap flow in subsequent years was reduced 13-16% (Wang et al. 2005). It is important to note that
25 elevated [CO₂] often substantially increases total tree leaf area, such that decreased E_t (transpiration
26 *per* leaf area) due to elevated [CO₂] may not fully compensate for greater tree leaf area;

1 subsequently, total water use per tree ($L d^{-1}$) may still be increased (Bobich et al. 2010; Tognetti et
2 al. 1999; Uddling et al. 2008). Ultimately, whole tree water flux in elevated $[CO_2]$ will be
3 dependent on both J_s and tree leaf area production.

4
5 *Why are nocturnal fluxes differentially affected by elevated $[CO_2]$ under wet and dry conditions?*

6
7 In order to determine why $J_{s,r}$ and $E_{t,r}$ are differentially influenced by elevated $[CO_2]$ in wet and dry
8 conditions, we examined the suite of environmental variables which affect water fluxes during the
9 day, including D , U , soil water content, wood density, and wood moisture. Radiation, which is
10 clearly a strong driver of water fluxes during the day, was not examined because it was zero after
11 19:00. There were no significant differences in wood density or wood moisture content. Wind
12 speed was similar in all chambers due to the continually blowing fans. Analyses of $J_{s,r}$ at the same
13 D (Fig. 5a) showed a marked decrease in $J_{s,r}$ in the ED treatment at the *same* value of D ; therefore,
14 D was not the main driver of differences in $J_{s,r}$ under elevated $[CO_2]$ compared with ambient $[CO_2]$.

15
16 The decrease in total night sap flux in ED compared with AD may be the result of biological
17 processes during the day. Less water was transpired in ED compared with AD, and E/A was lower
18 in the dry treatment (Fig. 3a). In the dry treatment, low day-time fluxes followed the same
19 reduction under elevated $[CO_2]$ as low night time fluxes. Although the exact nature of the
20 relationship between day-time and night-time fluxes is not clear in *E. saligna*, previous studies have
21 also shown that day-time fluxes are linked to night-time fluxes (Marks and Lechowicz 2007). Less
22 refilling of the tree trunk may occur because it requires less water to fill, as a result of the lower
23 volume of water transpired during the day compared with AD, and therefore less capacitance is
24 available. Or conversely, perhaps the tree uses less water during the day because the volume of
25 water refilled the previous night was smaller in ED than in AD. Regardless of which of these two
26 processes is dominant, the volume of water required to recharge the tree at night clearly influences

1 ψ_{pd} which is a strong indicator of plant water stress and a strong driver of water fluxes during the
2 day.

3
4 Higher values of total night sap flux in EW compared with AW may be explained by two possible
5 mechanisms. First, flushing leaves have higher nocturnal water fluxes than older leaves (Phillips et
6 al. 2010). Elevated CO_2 is known to influence leaf phenology (Lewis et al. 2003), and the
7 combination of wet soil and high CO_2 may have lead to plants in EW containing a higher
8 proportion of newly flushed leaves, despite a smaller leaf area, than in AW. Second, the role of
9 nutrient transport has been raised as a mechanism explaining nocturnal water fluxes, although the
10 evidence is equivocal (Howard and Donovan 2007; Howard and Donovan ; Scholz et al. 2007).
11 Nutrient transport would be more likely to occur in wet soil compared with dry soil because water
12 facilitates the transport of nutrients. Further, there is evidence that leaves grown under elevated
13 $[CO_2]$ have reduced nutrient concentrations compared with ambient $[CO_2]$ (Lewis et al. 2002;
14 Lewis et al. 2010), thereby requiring greater nutrient transport.

15
16 *Impact of elevated $[CO_2]$ on soil water status*

17
18 In the present study, a lower (more negative) ψ_{pd} for the ED trees compared with AD during the
19 severe drought period (Fig. 1) treatment was counter-intuitive, particularly as the ED trees had
20 lower leaf area. However, higher water stress (more negative ψ_{pd}) under elevated $[CO_2]$ was similar
21 to previous studies that reported lower ψ_{pd} in plants which had higher leaf area under elevated
22 $[CO_2]$ (Centritto et al. 1999). Similarly Atwell et al. (2009) reported greater water stress during
23 drought for eucalypts in elevated $[CO_2]$ compared with ambient $[CO_2]$ due to shallow rooting under
24 elevated $[CO_2]$. This suggests that some plants, and in particular eucalypts, grown under elevated
25 $[CO_2]$ may experience more water stress, because roots are more concentrated at shallower depths,

1 which leaves them vulnerable to drought stress when soil moisture declines in shallow soil (Atwell
2 et al. 2009; Duursma et al. 2011). Our findings generally contradicted the ‘water savings’ paradigm
3 associated with growth in elevated [CO₂] (Wullschleger et al. 2002). A second explanation for
4 greater water stress under elevated [CO₂] was that recharge of tree stems was lower under elevated
5 [CO₂] than ambient [CO₂] (Fig 2b). This lower volume of recharge may lead to a reduced degree of
6 refilling of tree stems under elevated [CO₂], generating a more negative Ψ_{pd} (Fig 1). In any case,
7 we found no evidence of amelioration of drought stress under elevated [CO₂].

8

9 *The response of E_n to D in elevated [CO₂]*

10

11 Our results are similar to those indicating that the impact of elevated [CO₂] on water fluxes depends
12 strongly on environmental conditions (Kellomaki and Wang 1998) and that the impact is higher at
13 high D (Kellomaki and Wang 2000). The present study demonstrates that these patterns hold for
14 some weather conditions (dry soil, high night-time D) but not others (wet soil or low night-time D).
15 Wang et al (2005) reported that, similar to the current study, at high D , differences between sap
16 flux density under ambient and elevated [CO₂] were higher compared with low D (presumably also
17 because there is more ‘signal’ and less ‘noise’ in sap flow when D is high). Similarly, Wullschleger
18 and Norby (2001) found that stand transpiration during the day was significantly lower under
19 elevated [CO₂] only when $D > 1.0$ kPa and radiation was $> 400 \text{ J m}^{-1} \text{ s}^{-1}$. It is important to note that
20 our findings apply to nocturnal water fluxes under elevated [CO₂], while previous studies have only
21 examined the impact of [CO₂] on day-time water fluxes.

22

23 *Stomatal conductance at night*

24

1 Meta-analyses have reported that g_d generally declines under elevated $[\text{CO}_2]$ (Medlyn et al. 2001).
2 Many studies have quantified g_n (see review by Caird et al. 2007), and both Phillips et al. (2010)
3 and Zeppel et al. (2010) demonstrated that g_n in eucalypts was primarily due to stomatal rather than
4 cuticular conductance, which was $5.6 \text{ mmol m}^{-2} \text{ s}^{-1}$ in *E. saligna* (Phillips et al. 2010). A reduction
5 in g_n of 20% in January ($P = 0.095$) occurred during the summer months, but not during wetter
6 months before irrigation was withheld (Feb, March, May, June, July, September, November, and
7 December; $P > 0.10$). Water limitation may decrease g_n (Dawson et al. 2007; Howard and Donovan
8 2007), although g_n is not always reduced under water limited conditions. In fact, g_n was observed to
9 be $> 20\%$ of g_d , even during drought conditions, in 10 different species in four deserts in North
10 America (Tissue, unpublished data).

11
12 Benefits of $J_{s,r}$ or g_n are suggested to include the refilling of xylem embolism at night to prevent
13 hydraulic failure (Bucci et al. 2004), the transport of nutrients (Dawson et al. 2007; Scholz et al.
14 2007); (but see Howard and Donovan, 2010) and refilling of stem capacitance (Wang et al. 2008),
15 which will prevent or minimise water stress (Caird et al. 2007). However, hydraulic redistribution,
16 which ameliorates water stress in plants in xeric environments (Brooks et al. 2002) is reduced under
17 nocturnal transpiration (Howard et al. 2009). Subsequently, if nocturnal fluxes are reduced and
18 therefore hydraulic redistribution is increased, water stress may be alleviated. Furthermore, global
19 circulation models predict that temperatures will rise, and in many areas precipitation may
20 decrease, leading to greater declines in water availability. Thus, the role of nocturnal fluxes and
21 water stress of plants under a changing climate is complex and requires further investigation.

22 23 *Conclusions*

24 This study on large trees, grown in water limited conditions for extended periods, reported the first
25 results of the impact of elevated $[\text{CO}_2]$ on nocturnal fluxes. During an extended drought period, *E.*
26 *saligna* saplings experienced reductions in $J_{s,r}$ in elevated $[\text{CO}_2]$ (by 32%), but increased $J_{s,r}$ by

1 28% under well-watered conditions. Furthermore, as D increased, the reduction in $J_{s,r}$ under
2 elevated $[\text{CO}_2]$ was higher than at low D . That is, although elevated $[\text{CO}_2]$ reduced nocturnal sap
3 flow during drought, these reductions are likely to become greater at high night-time D (over 0.5
4 kPa), and that nocturnal sap flow decreases as drought progresses. Contrary to our initial
5 hypothesis, elevated $[\text{CO}_2]$ trees did not experience less drought stress compared with ambient
6 $[\text{CO}_2]$ trees. Nocturnal water fluxes were significantly lower in the ED treatment compared with
7 the AD treatment, suggesting drought was not ameliorated by elevated $[\text{CO}_2]$ in the dry treatment.
8 As atmospheric $[\text{CO}_2]$ levels are elevated, nocturnal sap flow which refills tree stems may be
9 reduced, thereby increasing tree water stress and vulnerability to drought in future climates. These
10 findings will enable improved predictions of diel water fluxes and should be incorporated into
11 ecophysiological models to more accurately represent water and carbon fluxes under global climate
12 change.

13

14 *Acknowledgements*

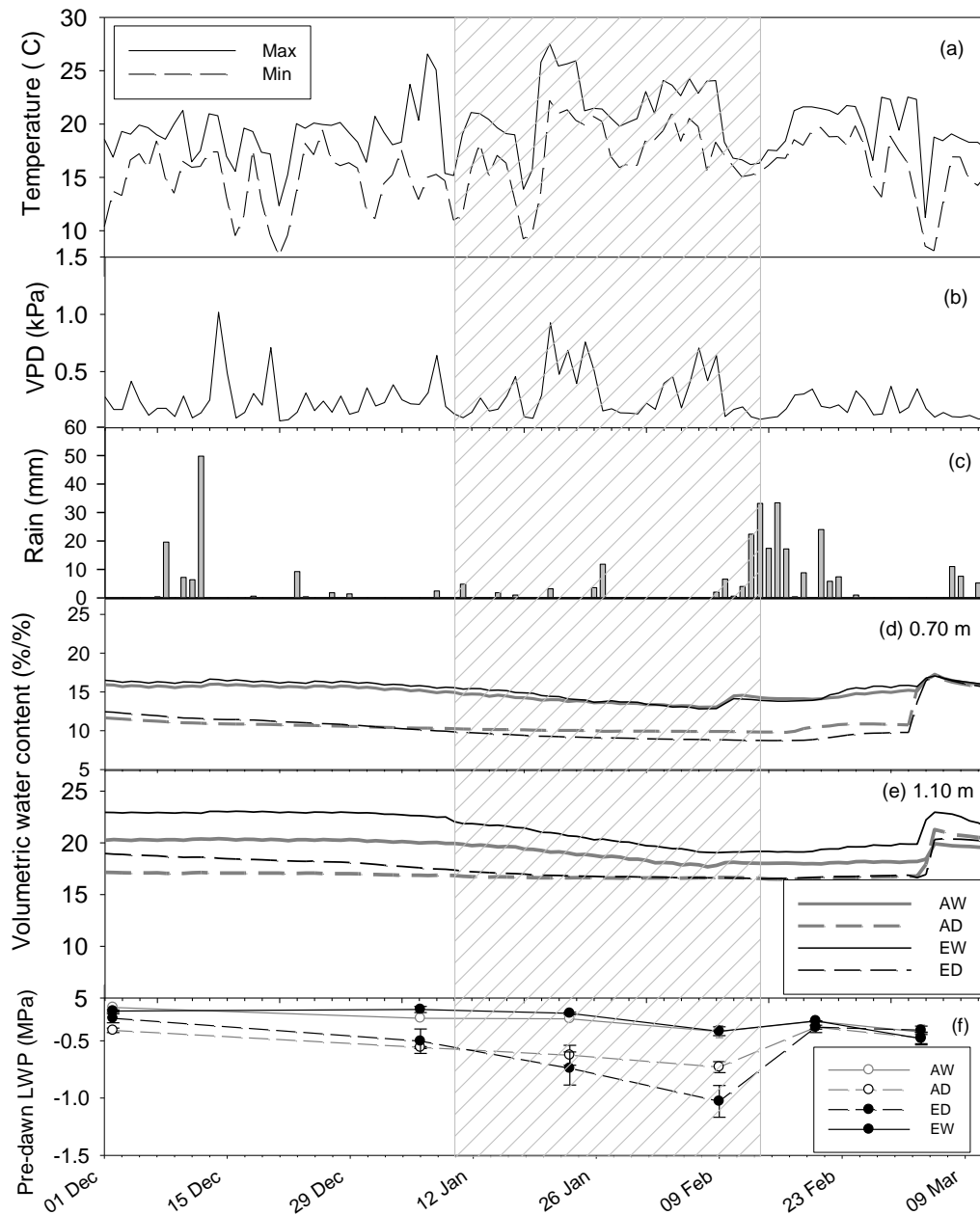
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17 Government's Department of Climate Change for the Hawkesbury Forest Experiment.

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3 Figure 1.

4 Environmental conditions during the study period, with temperature (minimum and
 5 maximum temperatures at night) (a), mean D at night (b), rainfall outside the
 6 chambers (c), volumetric water content (VWC) within each treatment at 0.70 m
 7 soil depth (d), VWC within each treatment at 1.10 m soil depth (e), and mean and s.e. of
 8 pre-dawn leaf water potential (Ψ_{pd}) of each treatment (f). The grey box represents
 9 the severe drought period (10 January to 14 February).

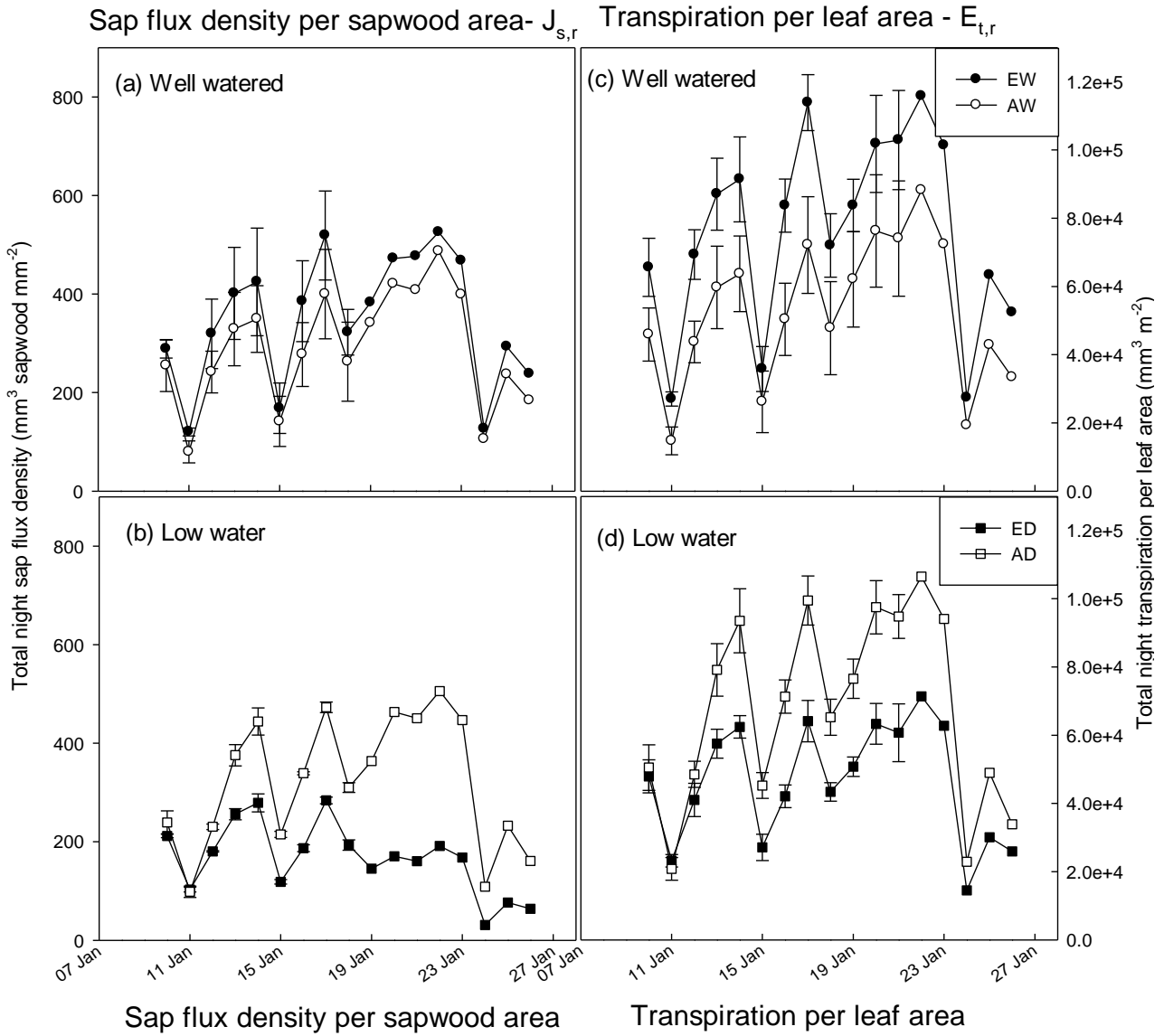
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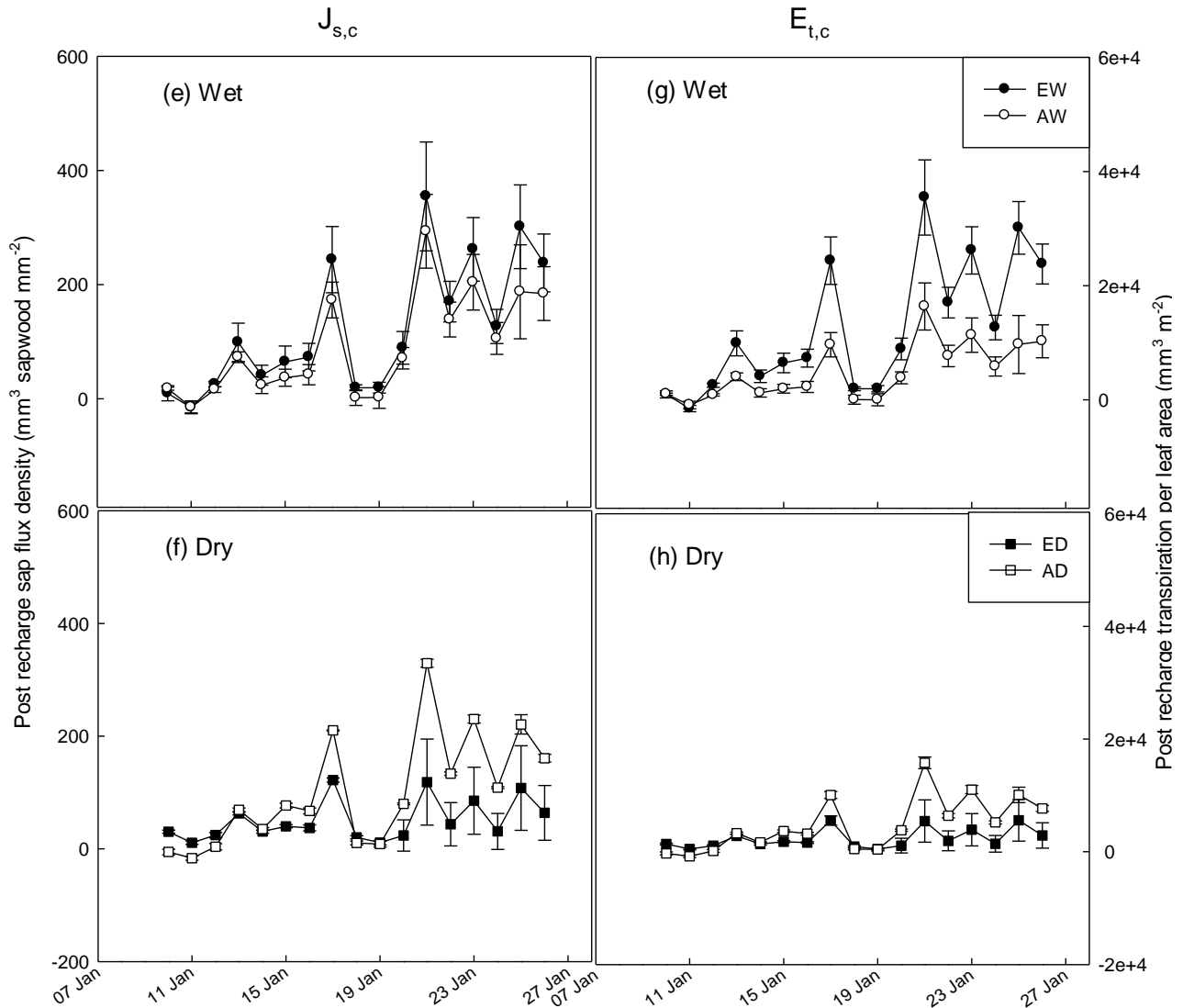
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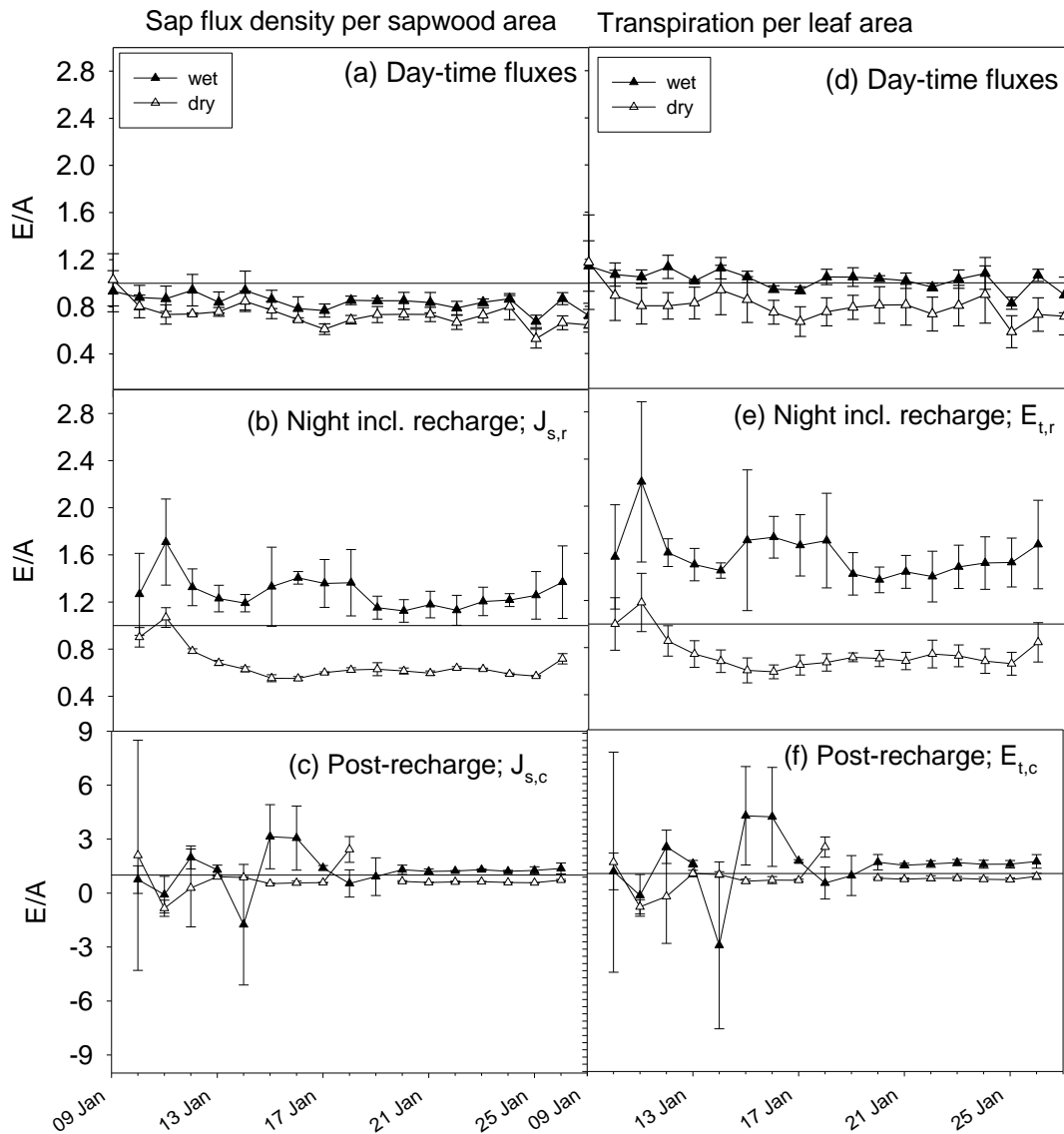
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Figure 2. Nocturnal sap flux density $J_{s,r}$ ($\text{mm}^3 \text{ night}^{-1} \text{ mm}^2$ sapwood area) of wet soil treatments (a) and dry soil treatments (b). Total transpiration per leaf area $E_{t,r}$ ($\text{mm}^3 \text{ night}^{-1} \text{ m}^{-2}$ leaf area) for (c) wet soil treatments and (d) dry soil treatments. Data points represent mean of each treatment and s.e. bars (only shown on the initial data points for clarity). Night is defined here as the hours between 19:00 and 05:00. Treatments are elevated [CO_2] and wet soil (EW); ambient [CO_2] and wet soil (AW); elevated [CO_2] and dry soil (ED); and ambient [CO_2] and dry soil (AD).



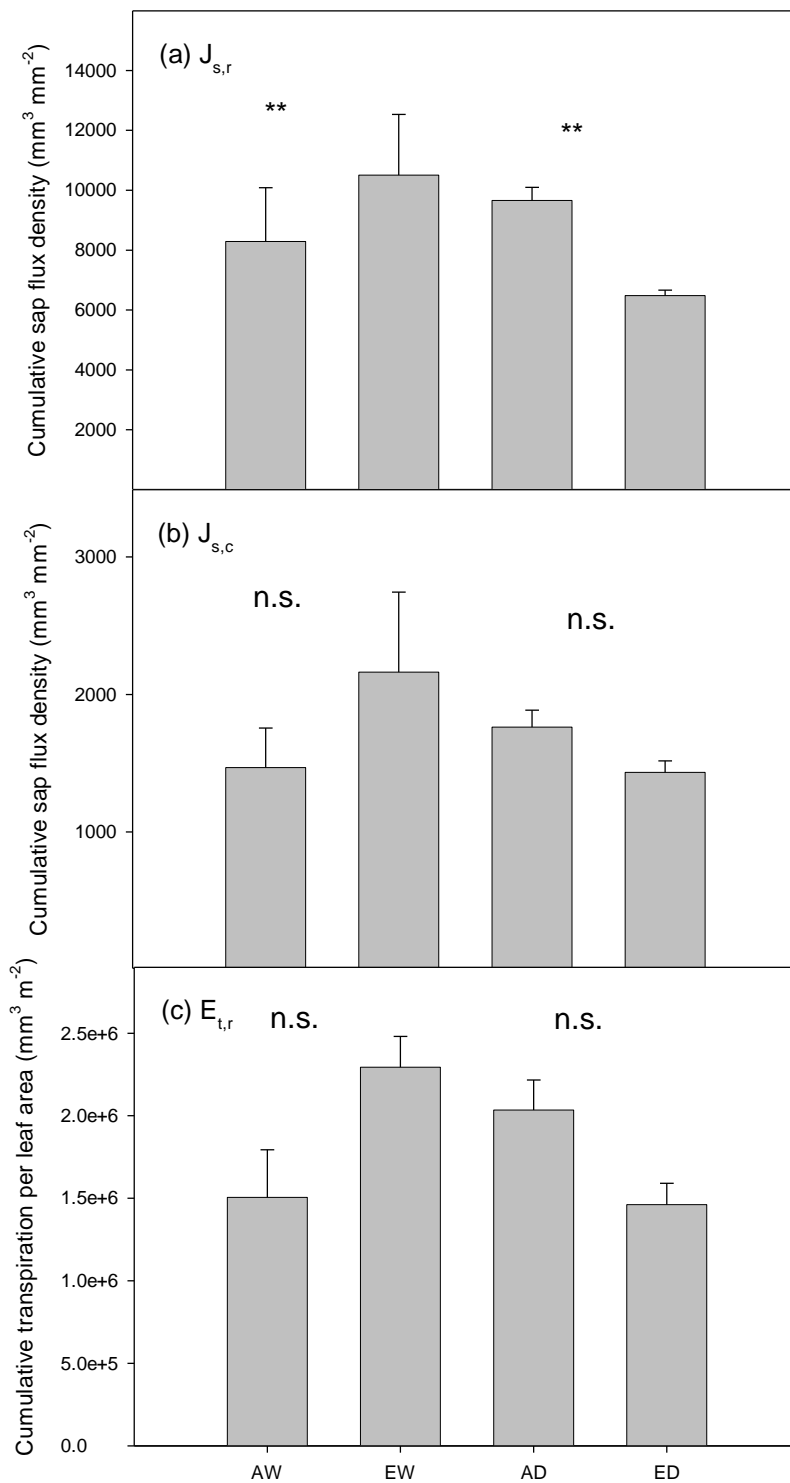
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3 Figure 2 e-h. Nocturnal sap flux density $J_{s,c}$ ($\text{mm}^3 \text{ night}^{-1} \text{ mm}^2$ sapwood area) of wet soil treatments
4 (a) and dry soil treatments (b). Total transpiration per leaf area $E_{t,c}$ ($\text{mm}^3 \text{ night}^{-1} \text{ m}^{-2}$ leaf area) for
5 (c) wet soil treatments and (d) dry soil treatments. Data points represent mean of each treatment and
6 s.e. bars. Night is defined here as the hours between 23:00 and 05:00. Treatments are elevated
7 $[\text{CO}_2]$ and wet soil (EW); ambient $[\text{CO}_2]$ and wet soil (AW); elevated $[\text{CO}_2]$ and dry soil (ED); and
8 ambient $[\text{CO}_2]$ and dry soil (AD).

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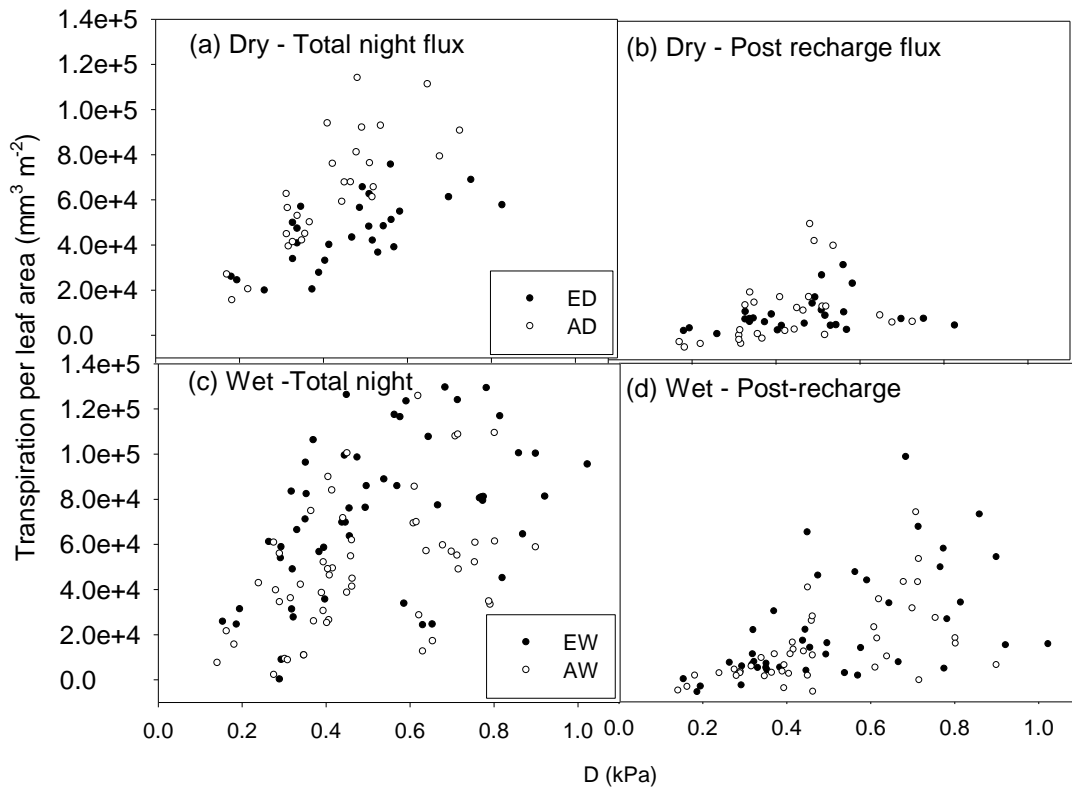
Figure 3. The ratio of elevated $[CO_2]$ to ambient $[CO_2]$ treatments (E/A) for sap flux density per sapwood area (a, b and c), and transpiration per leaf area (d, e and f). Dry treatments (ED/AD) are closed circles and wet treatments (EW/AW) are open circles. Data points represent the mean (and s.e.) of each treatment. Extremely large E/A ratios in the night excluding recharge period (c, f) are caused by the ratio of comparatively small absolute water fluxes between $J_{s,c}$ and $E_{t,c}$. The horizontal line represents $E/A = 1$.



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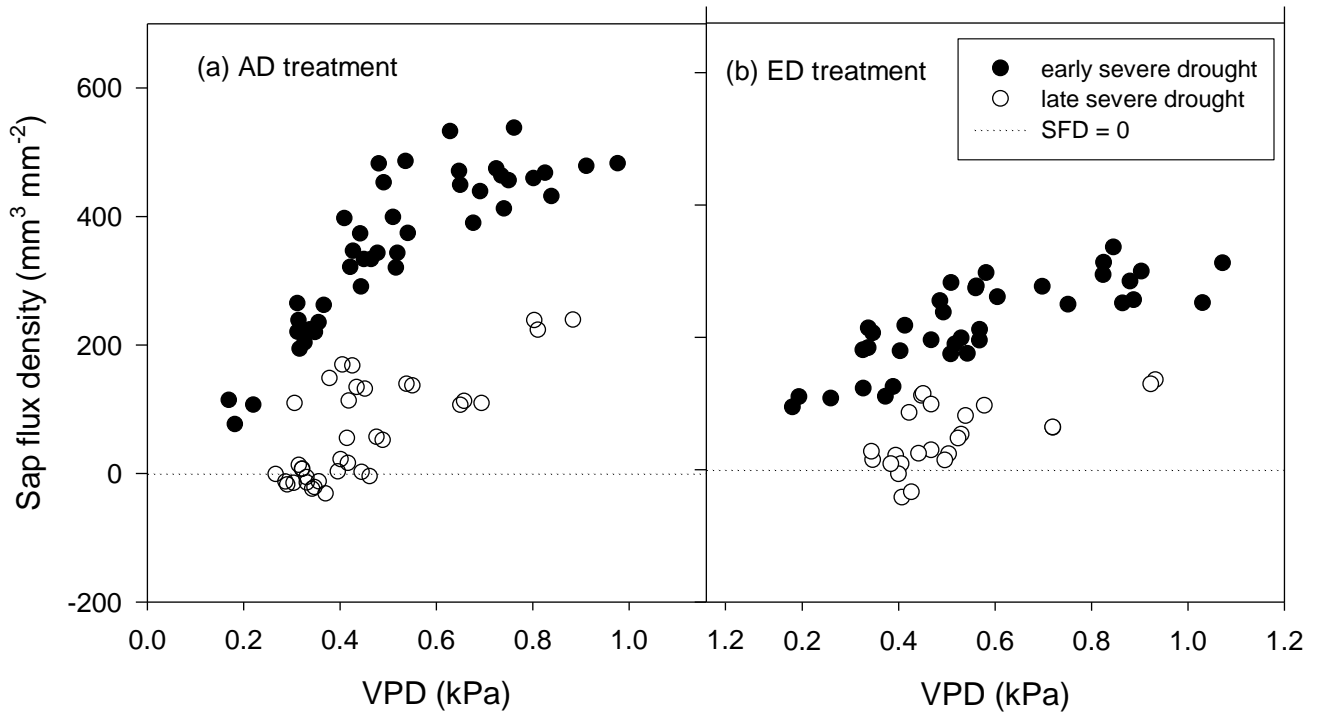
3 Figure 4. Total sum of water fluxes for the entire drought period for cumulative sap flux density
 4 during (a) the total night flux period $J_{s,r}$, and (b) the post-recharge period $J_{s,c}$ and (c) transpiration
 5 per leaf area ($E_{t,r}$). Treatments are elevated $[\text{CO}_2]$ and wet soil (EW); ambient $[\text{CO}_2]$ and wet soil

1 (AW); elevated [CO₂] and dry soil (ED); and ambient [CO₂] and dry soil (AD). Data represent
 2 means (and s.e) for each treatment. ** represents significant differences estimated using a Tukeys
 3 test.
 4



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 7 Figure 5. The response of transpiration (mm³ water per m² leaf area) to mean night *D* within dry
 8 treatments for total night flux period (19:00 to 05:00) in (a) dry and (c) wet treatments; and the
 9 post-recharge period (23:00 to 05:00) in (b) dry and (d) wet treatments. Mean night VPD is
 10 calculated as the mean *D* for the period 19:00 to 05:00 for ‘total night’ or 23:00 to 05:00 for ‘post-
 11 recharge’.

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3 Figure. 6. The relationship between total night sap flux density (normalised by sapwood area) for
 4 each night (19:00 to 05:00, $J_{s,r}$) for the D of each chamber for AD (a), and ED (b) treatments. Each
 5 point is one chamber per night. Within the severe drought period, 'early severe drought', (black
 6 circles) is defined as Jan 10 to Jan 23, and 'late severe drought', (open circles), is defined as Jan 24
 7 to Feb 14, based on distinct Ψ_{pd} in Figure 1.
 8