Changes in network connectance and temporal dynamics of gas exchange in *Citrus sinensis* under different evaporative demands

Gustavo M. Souza[1*], Rafael V. Ribeiro[1] and Steven M. Pincus[2]

[1]*Laboratory of Plant Stress Physiology, Dept. Biological Sciences, University of São Paulo - USP, CP 09, 13418-900, Piracicaba, SP, Brazil (Current address: Faculty of Agricultural Sciences, University of Oeste Paulista - UNOESTE Campus II, Rodovia Raposo Tavares km 572, 19067-175, Presidente Prudente, SP, Brazil);* [2]*Guilford, CT, 06437, USA.* **Corresponding author: gumaia@universiabrasil.net*

Stomatal aperture is an essential factor both in regulation of transpiration and net photosynthesis. This regulation is especially important in the response of plants to drought or to an increase in leaf-to-air vapor pressure difference (*VPD*); however, such a regulation is part of a complex dynamical environment, associated with multiple regulatory pathways. Accordingly, we studied the effects of *VPD* on gas exchange of *Citrus sinensis* via the evaluation of two complementary analytic approaches, to approach an understanding of the full scope of the system interactions. First, we used classical statistical methodologies, e.g., means, coefficient of variation, and linear correlation. Second, we used measures developed for more model-independent applications, Approximate Entropy (*ApEn*) to evaluate the irregularity or complexity of gas exchange time-series, and network connectance to evaluate changes in the extent of linkage among specified gas exchange parameters. The analyses of experiments carried out under constant environmental conditions in each *VPD* treatment (1.0, 2.0 and 3.0 kPa) showed a number of relatively subtle results of physiological consequence, such as differences in network connectance during the period of measurements at the same condition showing different patterns of gas exchange regulation. Additionally, *VPD* changes affect the dynamics of gas exchange by alterations in the irregularity of the time-series. These experiments highlight the endogenous and self-organized mechanisms that underlie the gas exchange process with further theoretical findings and possible practical applications.

Key words: approximate entropy, leaf-to-air vapor pressure difference, network modulation, time-series analysis.

Mudanças na conectância da rede e dinâmica temporal das trocas gasosas em *Citrus sinensis* sob diferentes demandas evaporativas: A abertura estomática é um fator essencial na regulação da transpiração e fotossíntese. Essa regulação é especialmente importante na resposta das plantas à seca ou ao aumento na diferença de pressão de vapor entre folha e ar (*VPD*). Tal regulação é parte de um complexo e dinâmico ambiente, sendo associada a múltiplas vias regulatórias. Neste trabalho, estudamos os efeitos do *VPD* sobre as trocas gasosas de *Citrus sinensis* mediante duas abordagens analíticas complementares, visando à compreensão das interações no sistema vegetal como um todo. Em primeiro lugar, utilizamos métodos clássicos de estatística, ex. médias, coeficientes de variação e correlação linear. Em seguida, utilizamos a entropia aproximada (*ApEn*) para avaliar a irregularidade ou complexidade de séries temporais de trocas gasosas, e conectância de rede para avaliar as mudanças nas relações entre parâmetros específicos de trocas gasosas. As análises dos dados coletados sob condições ambientais constantes em cada tratamento de *VPD* (1, 2 e 3 kPa) mostraram um conjunto de resultados de interesse fisiológico, como diferenças na conectância da rede de trocas gasosas durante o período de medidas na mesma condição de *VPD,* sugerindo diferentes padrões de regulação das trocas gasosas. Além disso, mudanças no *VPD* afetaram a dinâmica dessas trocas, alterando a irregularidade das séries temporais. Este estudo destacou os mecanismos endógenos e auto-organizados que são subjacentes aos processos de trocas gasosas com interessantes repercussões teóricas e possíveis aplicações práticas.

Palavras-chave: análise de séries temporais, diferença de pressão de vapor entre folha e ar, entropia aproximada, modulação de redes.

INTRODUCTION

Stomatal aperture is a determinant factor both in regulation of transpiration and net photosynthesis (Farquhar and Sharkey, 1982; Syvertsen and Lloyd, 1994; Jones, 1998). This regulation is especially important in the response of plants to drought or to an increase in vapor pressure deficit. For example, environmental constraints such as high vapor pressure deficit affect the plant gas exchange behavior, reducing stomatal conductance and then decreasing transpiration and net photosynthesis, for instance, as observed in *Citrus* spp (Khairi and Hall, 1976; Syvertsen and Lloyd, 1994; Brakke and Allen Jr, 1995; Medina et al., 1999; Veste et al., 2000). Moreover, variations in air humidity lead to changes in the stomatal behavior, disturbing the transpiration and net photosynthesis dynamic (Haefner et al., 1997; Mott and Buckley, 1998). By its rapid response to environmental changes, the control of stomatal opening plays a central role in the regulation of the internal water content, as well as the water use efficiency of plants (Levy, 1980; Jones, 1998).

However, such a regulation cannot be simply represented. Multiple pathways of regulation of stomatal aperture have been identified. They are based on the influence of internal CO_2, light intensity, light quality, temperature, air vapor pressure deficit and water (potential) in leaf tissues, PGRs (phytohormone growth regulators) concentration and cell sensitivity, and the biological clock (Farquhar and Sharkey, 1982; Zeiger et al., 1987; Erdei et al., 1998; Jones, 1998). In regard to such a complex reality, regulation of stomatal aperture appears as a highly complicated and multi-faceted process. These characteristics, of course, are hardly restricted to the regulation of stomatal aperture: live beings are typically dynamical non-equilibrium systems influenced by non-linearities, feedback, composite system features, and time delays (Nicolis and Prigogine, 1977; Hütt and Lüttge, 2002). Thus, it would not be unexpected that classical static measures such as moment statistics might fail to account for the full extent of the interactions that can emerge from the relationship between plants and their environment. In particular, in such a complex dynamical environment, some essential physiological correlates may show most vivid changes in statistics or measures apart from moments or linear correlation, prompting the present study.

According to Amzallag (2001), the effects of environmental disturbances on the gas exchange network may be evaluated through both changes in the variability of network elements and in the strength of the network connectance. It is generally accepted that variations about the average do not have biological significance, they would rather be experimental artifacts. However, there is strong evidence that the variability in biological measurements may have a biological significance (Trewavas, 1986; Green, 1996; Erdei et al., 1998; Amzallag, 1999 and 2001; Hütt and Lüttge, 2002). Changes in the variability level in a given parameter can be analyzed regardless of changes in the mean value of the parameter when the variability is normalized as a variation coefficient (CV) (Amzallag, 2001). The comparisons of CV values may indicate alterations in the system network, but they do not supply information on the nature of these changes. Thus, Amzallag (2001) suggests connectance evaluation of the network through the analysis of the correlation coefficients normalized. The correlation coefficient is considered not only a test of the significance correlation, but also as a measure of strength of the relationship (connection) between two parameters.

To assess changes in the temporal dynamics of gas exchange parameters, we utilize approximate entropy, *ApEn*, a model-independent statistic defined in Pincus (1991), with further mathematical properties and representative biological applications (Fleisher et al., 1993; Pincus and Singer, 1996; Pincus et al., 1996; Pincus and Kalman, 1997; Pincus and Singer, 1998; Pincus et al., 1998, Souza et al., 2004). Herein, we calculate *ApEn* for the overall time-series, for each physiological parameter, to assess possible changes in the dynamics of the recorded time-series. *ApEn* assigns a non-negative number to a sequence or time-series, with larger values corresponding to greater apparent process randomness or serial irregularity, and smaller values corresponding to more instances of recognizable features or patterns in the data.

Therefore, we characterized physiological responses to *VPD* changes in three ways. First, we compared the gas exchange parameter (i.e. transpiration, CO_2 assimilation, stomatal conductance and intercellular CO_2 concentration) averages in order to assess differences in each parameter in response to *VPD* changes. Second, we evaluated changes in the network composed by the relationships between the gas exchange parameters in order to assess differences in the network relationships as changes in the connectance between paired parameters and in the whole network. Finally, we quantify differences in the regularity of gas exchange temporal dynamics in each parameter to assess subtle changes in the underlying dynamics in response to the different *VPD* conditions.

As it was the first time that network connectance analysis and *ApEn* were used together to evaluate the responses of leaf gas exchange to different *VPD* conditions, some general

assumptions are needed for subsequent suitable interpretation of the results.

1) The number and strength of the connections among the elements in the network, i.e. connectance, of an organism is directly related to the stability of its system (Trewavas, 1986; Edelman and Gally, 2001).

2) Therefore, a greater connectance, *a priori*, may achieve higher system stability, although, according to the theoretical analysis of Gardner and Ashby (1970), there is a critical threshold for the system connectance level that could negatively influence the stability of the system. Thus, tighter network connectance could perform a higher level of control system, improving the system capacity to lead with external perturbations.

3) Adaptation is a process of general biological responses (outputs) to environmental stimuli (inputs) in order to enable organismic persistence (Gregorius, 1997).

4) Thus, changes in the system connectance, mostly connectance increases, could be considered as adaptive responses to environmental disturbances.

5) Temporal dynamics with higher irregularity (complexity) could be more efficient under environment constraints because of its intrinsic higher flexibility of response. According to van Voris and O'Neil (1980), Møller et al. (1998) and Souza et al. (2004b) complex dynamics may allow higher stability of biological systems under environmental disturbances, and *ApEn* can indicate properly changes in the level of complexity in such dynamical systems.

Accordingly, the objective of this study was to use new statistical tools in order to evaluate and quantify changes in gas exchange temporal dynamics of *Citrus sinensis* submitted to different leaf-to-air vapor pressure differences (*VPD*s), and changes in the gas exchange network modulation. To this end, we evaluated variations in the level of connection between physiological parameters of gas exchange, and we applied Approximate Entropy (*ApEn*) to each of the measurement variables considered, as described by Pincus (1991) and Pincus and Singer (1996) in order to quantify the irregularity or complexity of gas exchange time-series.

MATERIAL AND METHODS

Plant material: The measurements were carried out with 9 months-old sweet orange plants [*Citrus sinensis* (L.) Osbeck cv. Pera] grafted on 'Rangpur' lime [*Citrus limonia* (L.) Osbeck], grown in 20 L plastic pots under natural conditions at Piracicaba, SP, Brazil. Plants were watered until pot saturation daily and fertilized regularly.

Measurements of leaf gas exchange: For the measurements of gas exchange, we used an infrared gas analyzer (LI-6400, LiCor, Lincoln, NE USA) in an open system, with a dew-point generator (LI-610, LiCor, Lincoln, NE USA) attached in order to improve the leaf-to-air vapor pressure difference (*VPD*) control. The measurements were done using a 6 cm^2 clamp-on leaf cuvette. The LI-6400 was zeroed daily using H_2O- and CO_2-free air. The measurements were done in natural CO_2 air concentration (around 360 µmol.mol^{-1}).

On the early morning before measurements the plant of sweet orange 'Pera' was moved to the laboratory and kept well watered. A mature and fully expanded leaf was sealed into the gas exchange chamber in which the desired environmental conditions (temperature, *VPD* and light intensity) had been pre-adjusted. Leaves were submitted to photosynthetic photon flux density (*PPFD*) of 800 µmol.m^{-2}.s^{-1} and maintained at 30ºC (leaf temperature) in each *VPD* treatment. *PPFD* was maintained constant using an artificial quartz halide light source (LI-6400-02 LED light source, LiCor, Lincoln, NE USA) controlled with a quantum sensor located inside the leaf cuvette. Time-series of gas exchange in each *VPD* treatment, 1.0 (*VPD*1), 2.0 (*VPD*2), and 3.0 kPa (*VPD*3), were taken separately on different days. After an early adaptation of 1 h to each chamber condition, measurements were recorded in each 10 s from 9:00 a.m. to 4:00 p.m. Measurements of leaf gas exchange included: CO_2 assimilation (*A*, µmol CO_2.m^{-2}.s^{-1}), stomatal conductance (*gs*, mol H_2O.m^{-2}.s^{-1}), transpiration (*E*, mmol H_2O.m^{-2}.s^{-1}) and intercellular CO_2 concentration (*Ci*, µmol.mol^{-1}). The water use efficiency [*WUE*, µmol CO_2.(mmol H_2O)$^{-1}$] is the result of *A* normalized by *E*. These measurements were done in the same mature and completely expanded leaf.

Concerning the sources of measurement noises, according to Buckley et al. (1997), we can suppose minimal stomatal heterogeneity effects on gas exchange measurements. Furthermore, the noise in measurements (Pearcy et al., 1989) was evaluated with the empty leaf cuvette. This test showed that the noise due to the LI-6400 was very low (<2 % for *A* and <0.005 % for *gs*) and thus could be presently ignored. The coefficient of variation (total CV %, which represents the sum of variations of the air flow rate, CO_2 and water vapor differentials) of the LI-6400 was around 0.1 % during the measurements. Furthermore, the LI-6400 typically takes new measures every 0.75 s, which is suitable for the baseline time interval used in this study (10 s).

The experiments were performed five times (four times in the same plant and once in a different one), and gave

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qualitatively similar physiological results. All data presented here are from the most representative data set.

Data analysis: Data were submitted to ANOVA and the means of physiological parameters in different *VPD*s were compared by the Tukey test (p<0.05).

To assess changes in system network aspects, we evaluated the occurrence of differences in the system modulation of gas exchange when submitted to different *VPD*s via the concept and measurement of global connectance, *Cg* (Amzallag, 2001). Accordingly, to define connectance, first we specify a collection of paired variables of interest in the network. Next, we utilize the correlation coefficients (r) between each paired variable not only to test the significance of the correlation, but also as a measure of the strength of the relationship (connection) between the two parameters, by forming the z-transformation: z = 0.5 ln [(1 + r)/(1 - r)]. Finally, we define the network global connectance (*Cg*) of the specified collection of paired variables as the average of the absolute z-values calculated above (Amzallag, 2001). In this study, we calculated *Cg* for the following collection of paired variables: *A* x *gs*, *A* x *Ci*, *A* x *E*, *gs* x *E*, and *gs* x *Ci*, with results displayed in tables 3 and 4.

The irregularity of the time-series was assessed by *ApEn*. Two input parameters, a run length *m* and a tolerance window *r*, must be specified to compute *ApEn*. This parameter measures the logarithmic likelihood that runs of patterns that are close (within *r*) for *m* contiguous observations remain close (within the same tolerance width *r*) on next incremental comparisons; the precise mathematical definition is given in Appendix. The opposing extremes are perfectly regular sequences, (e.g., sinusoidal behavior, very low *ApEn*), and independent sequential processes (very large *ApEn*). It is imperative to consider *ApEn* (*m,r*) as a family of parameters; comparisons are intended with fixed *m* and *r*.

When m = 2, as is employed herein, we interpret *ApEn* as a measure of the difference between the probability that

runs of length 2 will recur within tolerance r and the probability that runs of length 3 will recur to the same tolerance. A high degree of regularity in the data would imply that a given (matched) run of length 2 would often continue with nearly the same third (next) value, producing a low value of *ApEn*.

ApEn evaluates both dominant and subordinant patterns in data; notably, it will detect changes in underlying episodic behavior not reflected in peak occurrences or amplitudes (Pincus and Keefe, 1992). Additionally, *ApEn* provides a direct barometer of feedback system change in many coupled systems (Pincus and Keefe, 1992; Pincus, 1994).

ApEn is nearly unaffected by noise of magnitude below *r*, a *de facto* filter level. *ApEn* is robust or insensitive to artifacts or outliers: extremely large and small artifacts have small effect on the *ApEn* calculation, if they occur infrequently. Both these points are evidently useful in clinical and experimental contexts, such as our present setting.

Further technical discussion of mathematical and statistical properties of *ApEn*, including mesh interplay, relative consistency of (*m,r*) pair choices, asymptotic normality under general assumptions, and error estimation for general processes can be found elsewhere (Pincus and Huang, 1992; Pincus and Goldberger, 1994). To develop a more intuitive, physiological understanding of the *ApEn* definition, a multistep description of its typical algorithmic implementation, with figures, is developed in Pincus and Goldberger (1994). Moreover, an extended discussion that covers a broad variety of both methodological aspects, plus considerably more background on the physiological meaning and scope of applicability of *ApEn*, can be found in Pincus (2000).

For the studies discussed below, *ApEn* values were calculated with widely established parameter values of *m* = 2, and *r* = 20 % SD (standard deviation) of the gas exchange time-series. Normalizing *r* to each time-series SD in this manner gives *ApEn* a translation- and scale-invariance (Pincus

Table 1. Mean values and coefficient of variation (CV %) of the overall time series (N = 2.200) of CO_2 assimilation (*A*), stomatal conductance (*gs*), intercellular CO_2 concentration (*Ci*), transpiration (*E*) and water use efficiency (*WUE*) of *Citrus sinensis* under three leaf-to-air vapor pressure differences (*VPD*).

VPD	A(μmol.m^{-2}.s^{-1})		gs(mol.m^{-2}.s^{-1})		Ci(μmol.mol^{-1})		E(mmol.m^{-2}.s^{-1})		WUE(μmol.mmol^{-1})	
	Mean	CV	Mean	CV	Mean	CV	Mean	CV	Mean	CV
1 kPa	2.29	21.40	0.034	20.59	235.35	6.05	0.369	20.05	6.24	13.78
2 kPa	2.77	22.02	0.030	20.00	189.19	6.79	0.592	17.74	4.64	6.03
3 kPa	1.70	34.12	0.017	23.53	193.55	12.61	0.511	25.24	3.22	14.60

Table 2. Mean values ($\pm$ SD) of the time series (N = 300), by stage, of CO_2 assimilation (*A*), stomatal conductance (*gs*), intercellular CO_2 concentration (*Ci*), transpiration (*E*) and water use efficiency (*WUE*) of *Citrus sinensis* under three leaf-to-air vapor pressure differences (*VPD*).

VPD	St*	A(μmol.m^{-2}.s^{-1})	gs(mol.m^{-2}.s^{-1})	Ci(μmol.mol^{-1})	E(mmol.m^{-2}.s^{-1})	WUE(μmol.mmol^{-1})
1 kPa	A	2.23 $\pm$ 0.16	0.029 $\pm$ 0.002	227.38 $\pm$ 6.48	0.308 $\pm$ 0.016	7.25 $\pm$ 0.33
	B	2.62 $\pm$ 0.11	0.040 $\pm$ 0.001	229.28 $\pm$ 4.06	0.421 $\pm$ 0.006	6.22 $\pm$ 0.28
	C	2.14 $\pm$ 0.06	0.039 $\pm$ 0.001	246.03 $\pm$ 2.09	0.428 $\pm$ 0.005	5.01 $\pm$ 0.13
2 kPa	A	2.67 $\pm$ 0.30	0.029 $\pm$ 0.003	190.41 $\pm$ 5.31	0.566 $\pm$ 0.052	4.72 $\pm$ 0.13
	B	3.43 $\pm$ 0.07	0.036 $\pm$ 0.001	181.37 $\pm$ 3.03	0.703 $\pm$ 0.009	4.88 $\pm$ 0.13
	C	2.62 $\pm$ 0.17	0.029 $\pm$ 0.002	185.87 $\pm$ 4.14	0.574 $\pm$ 0.033	4.56 $\pm$ 0.13
3 kPa	A	2.01 $\pm$ 0.07	0.019 $\pm$ 0.001	192.55 $\pm$ 7.88	0.586 $\pm$ 0.008	3.44 $\pm$ 0.10
	B	2.29 $\pm$ 0.05	0.021 $\pm$ 0.001	171.69 $\pm$ 3.61	0.634 $\pm$ 0.004	3.60 $\pm$ 0.07
	C	1.35 $\pm$ 0.19	0.013 $\pm$ 0.001	189.86 $\pm$ 8.37	0.422 $\pm$ 0.043	3.20 $\pm$ 0.14

*Time series were divided in three stages (column denoted St; each stage N=300 points), according to the gs dynamics of each *VPD*: (A) increasing stage, (B) stationary stage and (C) decreasing stage.

Table 3. Coefficient of correlation (r, p<0.001) between CO_2 assimilation (*A*), stomatal conductance (*gs*), intercellular CO_2 concentration (*Ci*) and transpiration (*E*), and global connectance (*Cg*) of theses parameters in the overall time series (N = 2.200) of *Citrus sinensis* under three leaf-to-air vapor pressure differences (*VPD*).

VPD	A x gs	A x Ci	A x E	gs x E	gs x Ci	Cg
1 kPa	0.813	-0.872	0.763	0.994	-0.472	1.379
2 kPa	0.989	-0.766	0.989	0.998	-0.690	2.102
3 kPa	0.993	-0.878	0.994	0.999	-0.825	2.413

et al., 1993), in that it remains unchanged under uniform process magnification, reduction, or constant shift higher or lower. Multiple previous studies that included both theoretical analysis (Pincus, 1991; Pincus and Keefe, 1992; Pincus and Goldberger, 1994) and biological applications (Pincus et al., 1993; Pincus et al., 1996; Morrison and Newell, 1996; Pincus et al., 1996; Christen et al., 1998; Pincus et al., 1998; Bruhn et al., 2000, Souza et al., 2004) have demonstrated that these input parameters produce good statistical reproducibility for *ApEn* for time-series of the lengths considered herein. In particular, one *ApEn* standard deviation $\pm$ 0.055 under very general conditions for *ApEn* for time-series of the lengths we analyze herein. Thus *ApEn* values that differ by 0.15 represent nearly 3 *ApEn* SDs, indicating true distinction with error probability nearly p = 0.001.

Because the time-series of all physiological parameters in the present experiments were markedly nonstationary,

ApEn was applied to the first-differenced gas exchange time-series. This is a standard statistical method to stationarize time-series and is applicable to a very broad class of models (Chatfield, 1989; Willians, 1997). In order to verify the appropriateness of this data transformation, the autocorrelation coefficients of the first-differenced time-series were calculated, and representative correlogram plots are shown (figure 2).

RESULTS

Effects of VPD on gas exchange: First, for each parameter, the (overall) measurements performed throughout the day were averaged, and compared for exposure to different leaf-to-air vapor pressure differences. The highest *gs* values were observed in *VPD* of 1 kPa (*VPD*1), whereas the net photosynthesis (*A*) and transpiration (*E*) exhibited the highest values in *VPD* of 2 kPa (*VPD*2) (table 1). Regardless the *VPD* treatment, *Ci* showed a reverse relationship with *A* (figure 1), suggesting that CO_2 uptake was higher than CO_2 influx, likely due to low *gs* values, when compared with the literature (Syvertsen and Lloyd, 1994). The *WUE* was the highest under exposure to *VPD*1, indicating that under this condition, a high efficiency in balance between water loss and CO_2 assimilation was maintained. Increase in atmospheric demand from 1.0 to 3.0 kPa, caused reductions in *A*, *gs*, *Ci*, and *WUE* of about 25.8, 50.0, 17.8 and 48.4 %, respectively. Nonetheless, a simple relationship between these parameters is not apparent. The decrease of *A* cannot be seen solely as caused by stomatal control because *Ci* in *VPD* of 3

Table 4. Coefficient of correlation (r) of the time series, by stage, between CO_2 assimilation (A), stomatal conductance (gs), intercellular CO_2 concentration (Ci) and transpiration (E), and global connectance (Cg) of these parameters of *Citrus sinensis* under three leaf-to-air vapor pressure differences (VPD).

VPD	Stages	A x gs	A x Ci	A x E	gs x E	gs x Ci	Cg
1 kPa	A	0.766[1]	-0.936[1]	0.762[1]	0.993[1]	-0.568[1]	1.437
	B	0.106	-0.809[1]	-0.003	0.861[1]	0.173	0.541
	C	0.725[1]	-0.501[1]	0.482[1]	0.808[1]	-0.256[1]	0.675
2 kPa	A	0.986[1]	-0.927[1]	0.987[1]	0.999[1]	-0.858[1]	2.343
	B	0.012	-0.907[1]	-0.200	0.781[1]	0.315[1]	0.620
	C	0.902[1]	-0.628[1]	0.897[1]	0.996[1]	-0.235[1]	1.405
3 kPa	A	0.508[1]	-0.943[1]	0.531[1]	0.988[1]	-0.704[1]	1.269
	B	0.449[1]	-0.890[1]	0.425[1]	0.946[1]	-0.101	0.851
	C	0.985[1]	-0.918[1]	0.984[1]	0.999[1]	-0.840[1]	2.290

*Time series were divided in three stages (N = 300), according to gs dynamic of each *VPD*: (A) increasing stage, (B) stationary stage and (C) decreasing stage.
[1]p<0.0001.

Table 5. Approximate Entropy (*ApEn*) of the first-differenced time series (N=2.200) of CO_2 assimilation (A), stomatal conductance (gs), intercellular CO_2 concentration (Ci), transpiration (E) and water use efficiency (WUE) of *Citrus sinensis* under three leaf-to-air vapor pressure differences (VPD).

VPD	A	gs	Ci	E	WUE
1 kPa	0.966	1.622	1.424	1.506	1.516
2 kPa	1.202	1.687	1.233	1.595	1.360
3 kPa	1.771	0.990	1.672	1.602	1.744

kPa (*VPD*3) was 2.27 % higher than in *VPD*2 (table 1). This result suggests that the internal CO_2 concentration was not the only constraint in decreasing the rate of photosynthesis.

However, the overall results presented in table 1 do not reflect short term fluctuations due to heterogeneous and distinct sub-epochs. The large CV values calculated here (table 1) suggest an important running variation in the parameters measured; this observation, in conjunction with inspection of Figure 1, suggested a more demarcated statistical evaluation (table 2). Accordingly, we next consider the temporal variation of the parameters measured.

The fluctuation of *gs* has been measured at short time intervals throughout the day (from 9:00 to 16:00). For several of the measurement variables under consideration (*gs*, *A*, *E*), in the three *VPD* conditions, three main stages may be distinguished: a first stage (A) of increasing values, followed by a second stage (B) with relative stability or mean stationarity in values, then by a third stage (C) of steady decrease in values (e.g., figure 1B for *gs*). The measurement variable *Ci* mirrors this evolution, although in reverse – it first decreases, then stabilizes, and finally increases (figure 1D). Thus, we separated the time-series into three successive

stages, the ascending, stationary, and decreasing stages respectively, analyzing each stage separately. As observed in figure 1, the duration of each one of these stages varies according to the treatment.

The duration of each stage, as estimated from the gs evolution is indicated in figure 1. Except for water use efficiency (which is a composite parameter), all the measured parameters showed a stage transition at approximately the same time. In performing this segmented stage analysis, we can more meaningfully verify via the correlation between gs and the other parameters the level of stomatal control on gas exchange.

To implement suitable statistical comparisons, both within and across stages, we compared samples of 300 data points for each stage. The short runs (N = 300) were sampled from the mid-section of each stage, in order to avoid possible boundary effects of the following stage. The mean values of all physiological parameters calculated within each stage confirm the tendencies previously described in figure 1 for *VPD*2 and for *VPD* 3, except gs in *VPD*1. As well, it is important to note that for *VPD*1 the stage C duration was the shortest among the stages, interestingly with a very sharp decreasing trend for *A*. Changes in *VPD* affected the span stages. Stage A in *VPD*1 was 148 min longer than in *VPD*3, while stage C in *VPD*3 was 175 min longer than in *VPD*1. Stage B (stationary) was longest in *VPD*1, and it was progressively shortened in *VPD*2 and *VPD*3. The *E* time-series had very similar (stage) time demarcations to the *gs* time-series. However, the decreasing stage C of the *A* time-series in the different *VPD*s tended to commence before stage C of *gs*, markedly in *VPD*1 when stage C of *A* started 167 min before, when compared with the *gs* stage C start (figure
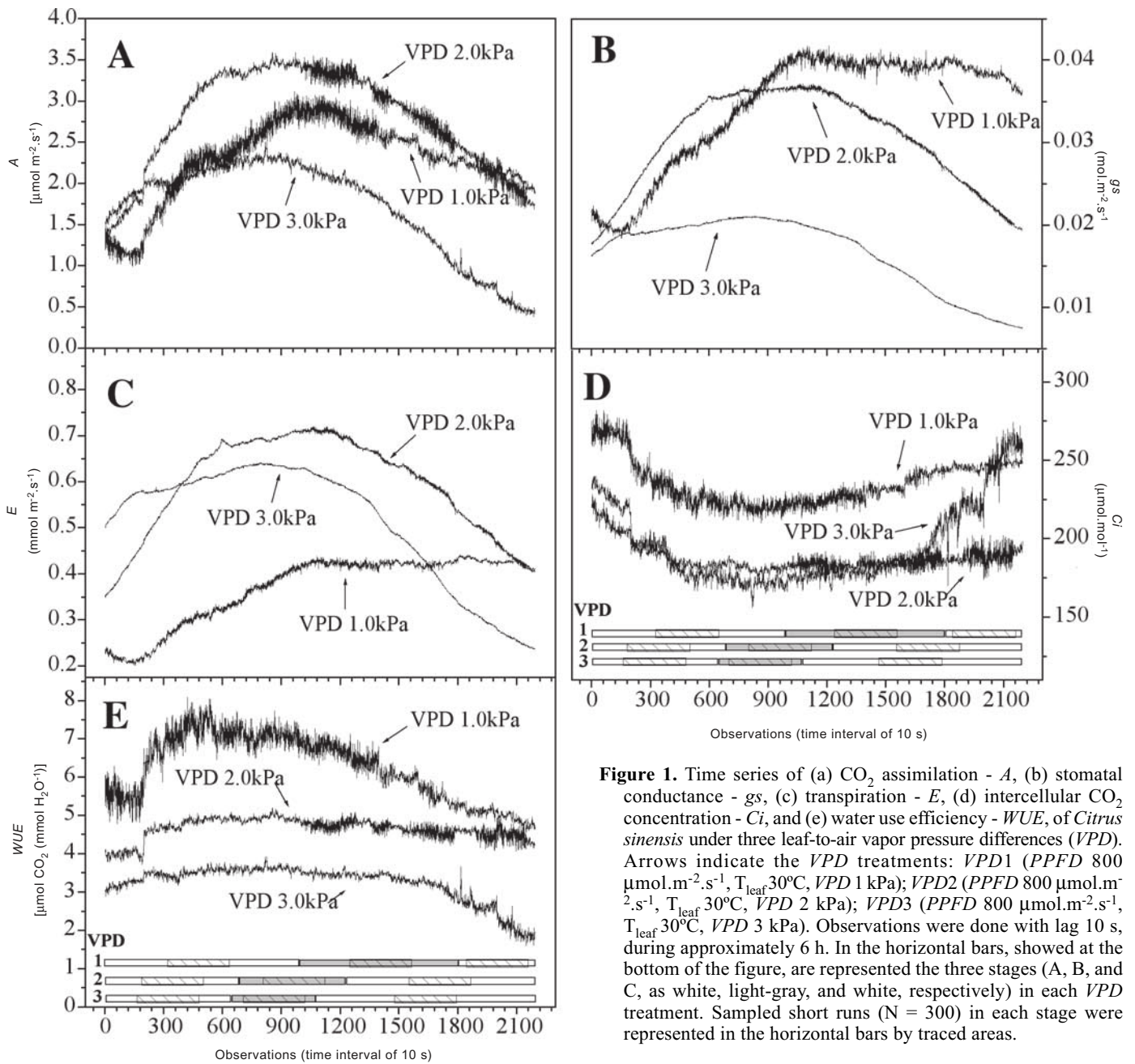
Figure 1. Time series of (a) CO_2 assimilation - *A*, (b) stomatal conductance - *gs*, (c) transpiration - *E*, (d) intercellular CO_2 concentration - *Ci*, and (e) water use efficiency - *WUE*, of *Citrus sinensis* under three leaf-to-air vapor pressure differences (*VPD*). Arrows indicate the *VPD* treatments: *VPD*1 (*PPFD* 800 µmol.m^{-2}.s^{-1}, T$_{leaf}$30ºC, *VPD* 1 kPa); *VPD*2 (*PPFD* 800 µmol.m^{-2}.s^{-1}, T$_{leaf}$ 30ºC, *VPD* 2 kPa); *VPD*3 (*PPFD* 800 µmol.m^{-2}.s^{-1}, T$_{leaf}$ 30ºC, *VPD* 3 kPa). Observations were done with lag 10 s, during approximately 6 h. In the horizontal bars, showed at the bottom of the figure, are represented the three stages (A, B, and C, as white, light-gray, and white, respectively) in each *VPD* treatment. Sampled short runs (N = 300) in each stage were represented in the horizontal bars by traced areas.

1). The stages of *Ci*, although inverse in direction as noted above, were similar to A stages insofar as timing is concerned, anticipated since *Ci* is the substrate of *A*.

Thus briefly summarizing the values of the physiological parameters among the three stages in the different *VPD*s (table 2), there was an increasing trend in *gs*, *A* and *E* values from stage A to stage B followed by a generally decreasing trend from stage B to stage C. *WUE* showed a decreasing trend from stage A to stage C in *VPD*1, while in *VPD*2 and 3 the highest *WUE* was observed in stage B. *Ci* tended to show the highest values

in stage A and the lowest ones in stage B. All differences reported here were statistically significant (p<0.05).

Effects of VPD on network connectance: Summarizing the overall time-series, *Cg* of the gas exchange network control showed a monotonic increase with *VPD* increase (table 3). There was a *Cg* increase in 55.65 % from *VPD*1 to *VPD*2, and the *Cg* from *VPD*2 to *VPD*3 increased by 16.25 %. The *gs* x *E* relationship was the most stable one across *VPD*s (r = 0.99, p<0.0001), due to the direct linkage between stomatal aperture and transpiration. However, such a simple link is
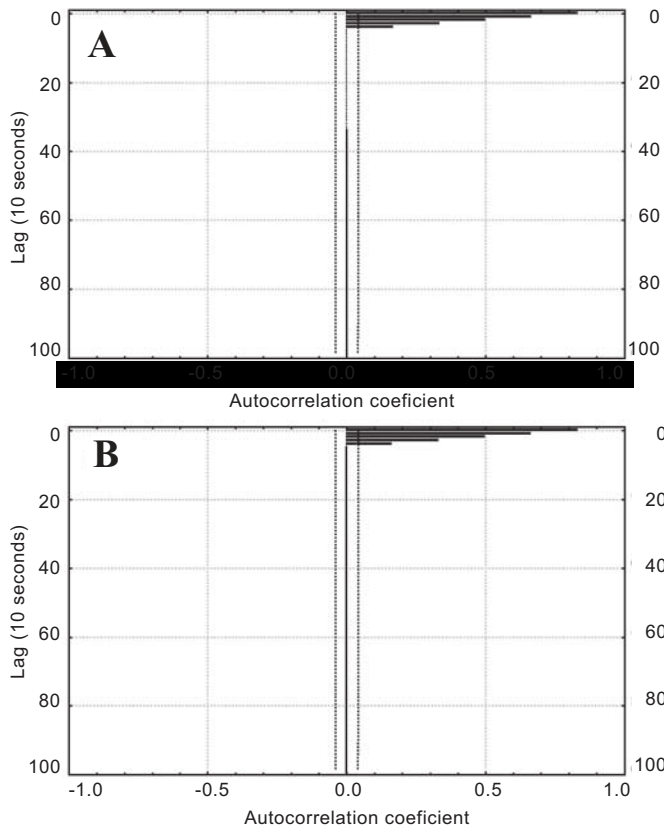
Figure 2. Correlograms of first-differenced time series for (a) CO$_2$ assimilation and (b) transpiration time series of *Citrus sinensis* under leaf-to-air vapor pressure difference of 2.0 kPa.

not observed between stomatal aperture and internal CO$_2$ content, since considerable variations in *r* values are observed between different treatments. Internal CO$_2$ concentration is both linked with stomatal aperture (CO$_2$ input) and photosynthesis (CO$_2$ output). The relationship between *Ci* and *A* also showed variations, but, surprisingly, the *r* value was the lowest under *VPD*2 (table 3).

Considerable variations in connectance are observed between different stages. In all the treatments, a minimum value of connectance is found during the second, stationary stage. It is due mainly to the elimination of relationships between stomatal aperture (*gs*, or *E*) with parameters related to photosynthesis (*A*, or even *Ci*) (table 4). One may notice that recovery of high connectance on the third stage is far from being achieved for *VPD*1, or even for *VPD*2. In contrast, the connectance is the highest at this third stage for *VPD*3. This suggests that a difference in regulation processes achieved towards the third stage between *VPD*1 or *VPD*2 treatments, and *VPD*3. *Cg* calculations with transformed (first-differenced) data were performed showing similar trends between stages in relation to *Cg* calculations from

original data (data not shown). Thus, in order to allow direct comparison to a number of previous studies, which have either always or usually also studied only the raw (non-transformed) data, we have retained the original *Cg* calculations in the text.

Effects of VPD on gas exchange dynamics: Although the *VPD* effects on gas exchange dynamics have exhibited differences in some experimental sets, the data shown below are representative of the general changes in gas exchange dynamics evaluated by *ApEn* calculations.

Despite the constant environmental conditions during measurements, the overall time-series of all physiological parameters were typically nonstationary (figure 1). Thus, *ApEn* was applied to first-differenced (D-1) gas exchange time-series. As observed in the correlograms of these D-1 time-series, the autocorrelation coefficients consistently showed a random-like fluctuation around a value of zero with time (lag), indicating that the first-difference was suitable in order to remove the nonstationary factors, i.e., approximately mean stationary. Furthermore, the correlation coefficients were practically zero for time-lags longer than about 50-60 s, showing the existence of the finite limit for significant auto-correlation. Correlograms of representative first-differenced time-series *A* (D-1) and *E* (D-1) in *VPD*2 are shown in figure 2. Similar results were obtained for time-series in *VPD*1 and *VPD*3.

The highest *ApEn* values in the overall time-series were typically found in *VPD*3, except for the *gs* time-series where the lowest *ApEn* value was found in *VPD*3. *ApEn* value of *A* time-series generally showed an increasing trend with increasing *VPD*. *Ci* and *WUE* time-series showed the lowest *ApEn* in *VPD*2, and the highest ones were found in *VPD*3. *ApEn* of *E* time-series were similar in the different *VPD*s, although a slight increasing trend from *VPD* 1 to 3 was observed (table 5). The *ApEn* results suggest that *A* was the most sensitive physiological parameter in order to capture changes in temporal dynamics.

DISCUSSION

The effects of increasing *VPD* on *A* and *E* have been associated with changes in *gs* (Khairi and Hall, 1976; Cowan, 1977; Farquhar and Sharkey, 1982). The partial stomatal closing in citrus in response to increasing *VPD* could be an evolutive adaptation to maintain the plant water status, mainly in regions of high atmospheric demand (Quick et al., 1992; Syvertsen and Lloyd, 1994). The *VPD* value that

caused stomatal closing in our study was 3.0 kPa; in other studies, these values were around 1.5 kPa (Sinclair and Allen Jr., 1982), 2.0 kPa (Khairi and Hall, 1976) and 3.0 kPa (Medina et al., 1998 and 1999).

However, as this study was carried out without water constraints with well-watered plants, a rigorous stomatal control of A and E was not observed, although gs responses to VPD, such as the accentuated decrease in $VPD3$, have been in agreement with natural *C. sinensis* responses to high atmospheric demands (Khairi and Hall, 1976, Syvertsen and Lloyd, 1994).

In spite of gs decreasing in $VPD2$ (12 %), E increased in 60 % (table 1). This effect in E may be understood by a straightforward relation with a higher atmospheric demand. From $VPD2$ to $VPD3$ gs decreased by 43 % while E decreased by only 14 % (table 1). This relative stability of E is probably explained by excessive stomatal opening, because of the well-watered condition, in relation to the low atmospheric demand in $VPD1$, furthermore an increasing in cuticular transpiration importance could be, in part, associated to an increase in VPD.

In the $VPD3$, A decreased by 39 % compared with $VPD2$. Although gs was low (just 0.017 mol.m^{-2}.s^{-1}) in $VPD3$, it was not the cause of A reduction because Ci was similar to $VPD2$ where A showed the highest values. This phenomenon is explained in part by stomatal patchiness resulting in overestimation of Ci (Cheeseman, 1991; Mott and Buckley, 1998; Cornic, 2000). Furthermore, these results could suggest that a biochemical limitation of CO_2 assimilation must be occurring, once Ci may be not limited. This endogenous decrease in A could be understood as an anticipated response (feedforward mechanism) to an eventual stomatal closing, promoted by a high VPD which could cause leaf dehydration and lead to maintenance of CO_2 storage for further photosynthesis support under stressed conditions (Raschke, 1987; Nobel, 1999; Luan, 2002).

WUE results support the lack of stomatal control of gas exchange because increasing VPD caused *WUE* decrease. In general, high VPDs under field conditions with water constraints are associated with *WUE* increasing (Larcher, 1995).

The control of stomatal movements is achieved by complex relationships among hormonal (ABA) and ionic balance, water movement and anatomical organization of the plant in response to environmental stimulation (Farquhar and Sharkey, 1982; Zieger et al., 1987; Jones, 1998). The regulation of photosynthesis is affected by stomatal behavior, and depends on an intricate metabolic network (Z-scheme,

Calvin-Benson Cycle) linked to source-sink dynamics (Farquhar et al., 1980; Farquhar and Sharkey, 1982; Jones, 1998). Probably, not only does gs affect both A and E, but these have a range of feedback effects on gs, either directly or indirectly. In addition, environmental factors may also affect any of these variables and the gain of the feedback loops (Jones, 1998). Therefore, the network findings in the present study, likely are not representing all agents involved in a "real" network.

Thus, we have two modes of regulation of gas exchange. The first is a double recontrol, so that A and E influence gs and gs also influences A and E, i.e., an autoregulation process. The first level of regulation in the relationship between A and gs may be doubled by another (heteroregulation), in which both gs and A are controlled by a third parameter.

The results showed changes in CV (table 1) in all parameters in the different VPD conditions. This variability indicated by CV values in the data settings may have a biological significance (Trewavas, 1986; Green, 1996; Erdei et al., 1998; Amzallag, 1999 and 2001; Hütt and Lüttge, 2002). Networks with a greater redundancy degree or degeneration, facilitate the maintenance of biological system homeostasis when disturbed by external factors (Trewavas, 1986; Edelman and Gally, 2001). For the overall series, the CV values were modified in parallel, suggesting that physiological parameters measured were probably linked, possibly in a star-like pattern, indicating a typical stress response (Amzallag, 2001). It is important to emphasize that under water restriction gs assumes a central role in the network of gas exchange modulation (Chaves, 1991; Jones, 1998). In a broad sense, a qualitatively similar pattern of organization is also observed between different stages in each VPD. As noted by Amzallag (2001), variability is not necessarily an undesirable experiment artifact. Rather, it is the transformation of the inherent noise by the biological system that provides information about its pattern of regulation.

When we consider the whole time-series, the increase in Cg with increasing VPD showed a continuous adaptive pattern of gas exchange regulation (Amzallag, 2001). However, the reduction in Cg at stage B in each time-series indicates an intrinsic non-monotonicity or discontinuity of the regulation pattern. The higher values of Cg in stages A and C (both nonstationary) in relation to stage B showed a higher gas exchange control, which could be related to the phenomenon of circadian cycle of aperture and stomatal closing observed even under constant environmental conditions (Kriedemann, 1968; Barrs, 1971; Hennessey and Field, 1991; Webb, 1998).

This phenomenon reinforces the endogenous or self-organized regulatory pattern of plants, which promotes a certain degree of autonomy in relation to the environment. Conversely, since higher levels of connectance are related to higher system stability (assumptions 1 – 4 in Introduction), the lowest values of *Cg* at stage B, independently of *VPD*, indicates that plants may be more susceptible to external stimuli at this period (Souza et al., 2004c), such as factors that cause the midday depression observed in citrus under field conditions (Brakke and Allen Jr., 1995).

In the time-series analyzed, the *VPD*3 treatment induced the highest *ApEn* values, except for *gs*. There was a general tendency towards increasing time-series irregularity from *VPD*1 to *VPD*3, but *ApEn* values of *VPD*2 showed an accentuated variation in each physiological parameter evaluated. This suggests that *VPD*2 was a transitory condition between a low atmospheric demand (*VPD*1) and a high one (*VPD*3). We propose that an increase in the control of the gas exchange regulation, such as observed in *VPD*3 (table 3), could afford a higher complexity of gas input and output dynamics through finer tuning, optimizing the gas exchange responses under higher atmospheric demand, accordingly to assumption 5 in the Introduction. The highest regularity of *gs* (indicated by the lowest *ApEn* values) in *VPD*3 could possibly be explained by the partial closing of the stomata, which could constrain their open-close movement limiting the range of aperture variation of stomatal pores (Nobel, 1999). Nonetheless, a similar finding was not seen for the *A* dynamic at *VPD*3, even given the low values of *A* in this condition, possibly due to the fact of *A* has been affected not only by the biophysical relations of gas exchange, but also because it is dependent on a complex enzymatic system of CO_2 carboxylation. Krempasky et al. (1993) observed different temporal dynamics in photosynthetic system ranging from periodic to chaotic in response to external stimuli. Thus, we suggest that photosynthesis may be the more suitable parameter to evaluate changes in temporal dynamics of plants under different environmental conditions.

Our results indicated that variations in *VPD* affected the temporal dynamic of gas exchange. However, because this is the first study that uses *ApEn* as a measurement of plant physiology regularity, some caution should be taken in relation to the interpretation of results. Future studies are necessary to validate functional correlates between alteration in patterns or dynamics and resultant interactions between plant status and environmental surroundings. Nonetheless, changes in *ApEn* have been shown to correlate with physiological and pathophysiological change in numerous and diverse settings (Fleisher et al., 1993; Pincus et al., 1993; Morrison and Newell, 1996; Pincus et al., 1996; Christen et al., 1998; Bruhn et al., 2000), which suggest that its use in plant research could be of considerable scientific utility when used to help to better understand plant physiological responses under stress conditions.

Our study showed that detailed quantitative analysis in plant biology experiments, particularly evaluations of temporal dynamics of physiological data, may be a more subtle problem than was generally previously considered. Because of general system nonlinearities and complex configurations and interactions, biological systems may be very sensitive to small changes in experimental conditions; thus we could *a priori* expect a broad range of responses to qualitatively similar stimuli. However, the results showed that citrus responses to *VPD* changes were coordinated by alterations in network connectance, which showed evidence of redundant modulation as indicated by changes in CV values (Amzallag, 2001).

Moreover, *VPD* changes affected the endogenous dynamics of gas exchange by alterations of stage lengths, and *ApEn* values (complexity of the time-series). These experiments in constant environment highlighted the self-organized mechanisms that drive the gas exchange process, indicating interesting biological endogenous modes of modulation and complex dynamics. Furthermore, the observation of different levels of network connectance throughout the day, such as the lower connectance in stage B, could be useful to crop management (e.g. to determine irrigation periods) or to scientific experimental designs (e.g. choice of the different periods of sensitivity to external stimuli).

Appendix

Mathematical definition of ApEn - Given N data points u(1), u(2), ... u(N), two input parameters, m and r, must be fixed to compute *ApEn* (denoted precisely by *ApEn*(m,r,N)). To define *ApEn*, first form vector-sequences x(1) through x(N-m+1) from the {u(i)}, defined by x(i) = [u(i), ... , u(i+m-1)]. These vectors represent m consecutive u- values,

commencing with the ith point. Define the distance $d[x(i),x(j)]$ between vectors $x(i)$ and $x(j)$ as the maximum difference in their respective scalar components. Use the sequence $x(1)$, $x(2)$, ... $x(N-m+1)$ to construct, for each $i \leq N-m+1$, $Ci^m(r) =$ (number of $x(j)$ such that $d[x(i),x(j)] \leq r$) / $(N-m+1)$. The $Ci^m(r)$'s measure <u>within a tolerance r</u> the regularity, or frequency, of patterns similar to a given pattern <u>of window length m</u>. Next, define $\Phi^m(r)$ as the average value of ln $Ci^m(r)$, where ln is the natural logarithm. We define approximate entropy by $ApEn(m,r,N) = \Phi^m(r) - \Phi^{m+1}(r)$.

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