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Occurrence, incidence and associations among fungal pathogens and *Agrilus auroguttatus*, and their roles in *Quercus agrifolia* decline in California

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Summary

Synchronous decline of oak (*Quercus* spp.) trees in woodlands has been described in Europe and eastern North America as a complex interaction of stressors that predispose, incite or contribute to tree death. This study presents a 2-year (2010–2011) assessment of the role of pathogens in coast live oak (*Quercus agrifolia*) woodlands in southern California where oak mortality occurs in locations that are infested and uninfested by the goldspotted oak borer (GSOB, *Agrilus auroguttatus*). Cumulative coast live oak mortality was not significantly different between sites and was weakly correlated with *Diplodia corticola* and GSOB incidence and negatively correlated with annual relative humidity. Multiple logistic regression models explained the presence of individual fungi or GSOB at the tree level. Fisher's exact test analysis determined that the presence of *D. corticola, Fusarium solani, Dothiorella iberica, Cryptosporiopsis querciphila* and *Diatrypella verrucaeformis* were each related to origin of sample location on tree, and *C. querciphila* was additionally related to symptom type on the bole. Multiple linear regression models showed high correlation between environmental variables and plot-level incidence of both GSOB and *D. corticola*. Disease incidence (DI) for *D. corticola* was highest in GSOB-uninfested locations. Jaccard index of association (*J*) showed that *D. corticola* was negatively associated with the presence of GSOB, *F. solani* and *C. querciphila.* Results suggest that oak decline in California is an example of a complex syndrome involving strong regional differences in factors that are associated with the problem.

1 Introduction

Synchronous decline of oak (*Quercus* spp.) trees has been described in woodlands across Europe and eastern North America (Swiecki and Ufnalski 1998) as a complex interaction of stressors that predispose, incite or contribute to tree death (Manion 1981; Führer 1998; Pedersen 1998). The ecological and economic importance of oaks in these regions (Sánchez et al. 2003; Saxena and Singh 1984; Pausas et al. 2006) has led to studies of these potential stressors (pathogens, pests, drought, pollution and other anthropogenic influences) and construction of descriptive models of their interactions (Thomas et al. 2002).

Since 2002, large-scale oak die-off of two red oaks, *Quercus agrifolia* and *Q. kelloggii* (subgenus *Erythrobalanus*), in southern California was observed in San Diego County and in 2008 was linked to larvae of the goldspotted oak borer (GSOB, *Agrilus auroguttatus*), a new pest in California that disrupts cambium and nearby phloem tissues in the bole (Coleman and Seybold 2008). Symptoms preceding mortality include crown thinning, dense feeding galleries of *A. auroguttatus* and patches of necrosis in the phloem that exude a dark reddish-brown sap.

However, *Diplodia corticola,* a canker pathogen that is a known contributor to the decline of cork oak (*Q. suber*), and other oak species throughout Mediterranean Europe (Luque and Girbal 1989; Luque et al. 2000, 2008; Sánchez et al. 2003; Alves et al. 2004) was also isolated from branch and bleeding cankers on the trunk of *Q. agrifolia* in locations both infested and non-infested by GSOB (Lynch et al. 2010, 2013a). Pathogenicity tests showed that *D. corticola* kills coast live oak seedlings, colonizes the taproot under glasshouse conditions and causes bleeding on mature field trees and inoculated seedlings (Lynch et al. 2010, 2013a). The likelihood of *D. corticola* being an exotic pathogen is explored in the study by Lynch et al. (2013a), but needs to be investigated for confirmation. In addition, several other newly recorded fungal pathogens of coast live oak in California were recovered from tissues associated with the aforementioned symptoms and were shown to cause these symptoms in pathogenicity tests (Lynch et al. 2013a,b). Species include *Diplodia agrifolia, Dothiorella iberica, Fusarium solani, Diatrypella verrucaeformis* and *Cryptosporiopsis querciphila*; *D. agrifolia* and *C. querciphila* were described as new species from this outbreak (Lynch et al. 2013a,b).

There had been no systematic surveys to assess the role of pathogenic fungi on declining oaks within GSOB-infested and GSOB-uninfested forests of southern California. Given that *D. corticola* co-occurs with multiple other fungal pathogens in Europe where oak decline has been documented (Luque and Girbal 1989; Luque et al. 2000, 2008; Sánchez et al. 2003; Alves et al. 2004) and that there appeared to be areas 20 miles to the northwest, beyond the current GSOB outbreak, where canopies of coast live oak were in sharp decline (T. A. Scott, personal observation), the nature of the decline and known behaviour of the pathogens recovered warranted such an investigation.

The objectives of this study were to establish plots in coast live oak forests throughout San Diego and Riverside counties in California where GSOB was well established, recently established or absent (i) to determine distribution, establishment

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and incidence of fungi pathogenic to oak in relation to GSOB occurrence and (ii) to assess how these agents may be influencing mortality of *Q. agrifolia* in southern California.

2 Methods

2.1 Study area and plot establishment

In southern California, *Q. agrifolia* occurs from sea level to approximately 1500-m elevation, typically in areas with >50 cm precipitation per year, but below areas of heavy snowfall (Scott 1991; Allen-Diaz et al. 2007). Coast live oak-dominated, mixed-evergreen deciduous forests and woodlands in San Diego and Riverside counties with tree mortality were surveyed in areas representing three GSOB infestation levels: infested, uninfested and recently infested (on the advancing margin of the infestation) (Table 1). The region experienced two recent multiple year droughts, in 1999–2004 and 2006–2007 (http://wwwcimis.water.ca.gov/cimis/data.jsp). The former included the lowest precipitation (3.02 inches) of any water year (July–June) since 1849– 50 (Isla et al. 2004). About 45% of oak-associated woodlands/forests were inside the perimeters of two exceptionally large wildfires in 2003 and 2007 (http://frap.cdf.ca.gov/data/frapgismaps/select.asp, T. A. Scott unpublished data).

Between December 2009 and May 2010, a total of 45 variable radius plots were established, 5 plots at each of nine locations (Table 1). Plots were at least 200 m apart, at random compass bearings from the plot centres, with all plot locations recorded using global positioning system (GPS) equipment (5–10 m accuracy). Percentage maximum solar radiation input was calculated for each plot from field-measured slope and aspect using equations provided by Buffo et al. (1972). Slope position (summit/ridgetop/plateau, shoulder, backslope, footslope, toeslope, valley bottom, alluvial flat) and plot history (natural disturbances, cutting, cattle disturbance) were recorded.

Variable plot radius ≥12.6 m (1/20 ha) was chosen to account for variable stand density across landscapes to achieve at least 10 trees per plot with ≥30 cm diameter at breast height (d.b.h), which is the size that GSOB will routinely infest in outbreak areas (Coleman et al. 2011). All trees within plots with ≥1.0 cm d.b.h. were measured and mapped (by azimuth and distance from the plot centre) and designated a crown position (stump, stob, understory, intermediate, codominant, dominant or emergent). For each tree, health, presence and severity of GSOB and non-GSOB exit holes, staining, woodpecker activity, bark cracking and other pests were recorded in 2010 and repeated for 2011. Health and trunk symptom assessments were determined by rating scales following protocols of Coleman et al. (2011). Presence and categories of fire scars were also noted for each tree (0 = absent; 1 = Basal fire scar; 2 = Fire scar moved up the bole; 3 = Charred).

2.2 Sampling

2.2.1 Tree selection

To determine GSOB and fungi colonizing trees, up to six trees per plot ≥30 cm d.b.h. were randomly selected for intensive trunk sampling. One trunk sample from all trees exhibiting bleeding/staining symptoms was collected to determine disease agents. To account for GSOB preferentially colonizing the base of oak boles (Coleman et al. 2011), intensive sampling of tissues with target symptoms on the tree trunk was from two different heights: one set within the first 1 m from the ground, and a second set up to 3 m above this height. For each tree position, tissues were collected with target symptoms of staining/bleeding, GSOB exit holes and non-GSOB insect exit holes, as available. Branches or twigs with symptoms of shoot or branch dieback were sampled from all trees. In 2011, tissues with new presentations of the symptoms on the six selected trees and additional tissues from staining symptoms on other trees were collected for fungal isolations. Trunk tissue of one asymptomatic tree per plot was also collected.

Table 1. Name, acronym, and county of survey sites in southern California where plots were located, and presence of the goldspotted oak borer (GSOB) and stand characteristics.

Site name	Site ID	County	GSOB presence[1]	Basal area (m^2/ha) ± SE[2]	Tree density (ha^{-1}) ± SE[2]
Marian Bear Regional Park	MBRP	San Diego	+	35.3 ± 2.2	344.8 ± 25.0
William Heise County Park	WHCP	San Diego	+	45.0 ± 2.0	247.6 ± 61.0
Samataguma Private Property	SMTG	San Diego	+	30.0 ± 1.8	127.9 ± 18.0
Pine Creek, Cleveland National Forest	CNFPC	San Diego	+	21.1 ± 1.2	127.6 ± 27.0
Wilderness Gardens Preserve	WGP	San Diego	−	149.6 ± 1.8	270.0 ± 64.0
Santa Rosa Plateau	SRP	Riverside	−	23.2 ± 1.0	153.4 ± 41.0
San Timoteo Canyon	RLC	Riverside	−	28.5 ± 2.8	88.6 ± 19.0
Santa Ysabel Preserve	SYP	San Diego	Margin	118.4 ± 12.0	220.0 ± 24.0
Mataguay Scout Camp	MSC	San Diego	Margin	54.9 ± 2.2	143.2 ± 34.0

[1]GSOB Presence: Site Infested by GSOB (+); Site Non-Infested by GSOB (−); Site Infested by GSOB but on the advancing margin of the infestation (Margin).
[2]SE, standard error.

2.2.2 Specimen collections

2.54-cm-diameter pieces of symptomatic bark with underlying sapwood were extracted using a sterile plug cutter (2.54 cm diameter). Holes were filled with wooden dowel pieces (2.54 cm diameter) and covered with wound sealant. Crown symptoms were collected using pruning shears or saws that were sterilized in 95% ethanol between samplings.

2.2.3 Isolations

The outer surfaces of crown samples were washed in laboratory with sterile deionized water to remove organic debris, briefly flamed and removed with a paring knife. Samples were cut in half to access uncontaminated tissue, and pieces on the leading margin of necrotic tissue were plated onto potato dextrose agar amended with 1% tetracycline (PDA-tet), pimaricin–ampicillin–rifampicin–pentachloronitrobenzene agar (PARP, a *Phytophthora* selective medium), 1% malt extract agar amended with 200 ppm cycloheximide and 100 ppm streptomycin sulphate (CSMA, selective medium for fungal species associated with ambrosia beetles), water agar (WA) and nutrient agar (NA). The presence of oomycetes in the soil was determined using baiting techniques and soil collection procedures (Fichtner et al. 2007). Soil was collected from the base of up to two trees per plot that exhibited staining. Cultures were incubated at 25°C and after 3–5 days, recovered fungi were subcultured for molecular identification and long-term storage. Fungal species identities were determined morphologically and using molecular and phylogenetic analyses (Lynch et al. 2013a,b). Pathogenicity of the most frequently recovered fungi was determined by confirming Koch's postulates (Lynch et al. 2013a,b).

2.4 Statistical analyses

Statistical analyses were performed using SAS (1995) (Version 9.3; SAS Institute, Cary, NC).

2.4.1 Pathogen and GSOB incidences and tree mortality

Proportion of trees with the presence of each fungal pathogen and GSOB was calculated to determine their incidences on a plot and site level. For fungal pathogens, incidence was determined based on recovery of fungi from live trees. However, incidence of GSOB was determined based on signs observed on all trees, live or dead. Cumulative oak mortality percentages were calculated for each plot based on all standing trees, living and dead. The mean cumulative oak mortality for each site was calculated in 2010 and 2011. Incidences and mortality rates at the plot level were used in the explanatory models (see below). Differences in incidences and mortality between sites were determined for 2011 using one-way analysis of variance (ANOVA). Comparisons for mean pairs were made using student's *t*-test ($\alpha = 0.05$). Within sites, differences between 2010 and 2011 were determined using chi-squared analysis.

2.4.2 Co-occurrence of agents

To assess the degree to which all pathogens, GSOB, the flat-headed apple tree borer (*Chrysobothris femorata*) and western oak bark beetle (*Pseudopityophthorus pubipennis*) occupy the same habitat at the plot level, association analysis was employed (Greig-Smith 1983; Ludwig and Reynolds 1988; Kent and Coker 1992; Turechek 2004). The probability of agents occurring together in the population of plots as sampling units was measured with the Jaccard index of association (*J*) (Greig-Smith 1983; Ludwig and Reynolds 1988; Kent and Coker 1992; Turechek 2004). To test if the observed Jaccard index is significantly different from the value calculated under the assumption of independence, a normal distribution is assumed for the index estimated by *J* (the observed Jaccard value). Standard error was estimated using a jackknifing procedure so that a z-test could be used to calculate a p-value. The jackknife procedure was from R (Canty and Ripley 2011).

2.4.3 Disease and mortality modelling

A stepwise multiple linear regression analysis that used stand structure, composition and environmental variables was used to develop a model for coast live oak communities to explain *D. corticola* and GSOB incidence and coast live oak mortality in a plot. Long-term averages of environmental data (annual precipitation, relative humidity, and maximum and minimum temperature from 1971 to 2000) were obtained from the PRISM Climate Group, Oregon State University, as based on the 'Parameter-elevation Regressions on Independent Slopes Model' (www.prism.oregonstate.edu). Number of years since the last fire data were obtained from individual reports given by respective land managers (listed in acknowledgements). The stepwise criterion was run with $p \leq 0.05$ for *F* for variable entry. A check of colinearity in the regression model was performed employing leverage plots and bivariate scatterplots. For parametric analyses, assumptions of normality and homogeneity of variances were checked and met.

Multiple logistic regression analysis was used to determine variables correlated with pathogen and GSOB presence on a tree. Various transformations of the data were subjected to multivariate logistic regression to obtain the most parsimonious model of disease or pathogen presence or absence in the sampling unit. All multivariate logistic models followed a similar protocol (Sharma 1996; Hosmer and Lemeshow 2000; Quinn and Keough 2002). Each explanatory variable was subjected to a univariate logistic regression to determine appropriateness for the model. The response variables (pathogen and GSOB presence) were tested against the explanatory variables separately, and a model was developed for each independently.

A stepwise logistic regression with a cut-off of $p < 0.05$ was used for variable selection. Those selected by the stepwise regression were examined for their contribution to the overall model by examining their likelihood ratios statistic and by comparing their estimated coefficient to the estimated coefficient from the model containing only that variable, and to the estimated coefficients of the model that went into the stepwise regression (Hosmer and Lemeshow 2000). The model(s) that resulted from this process was checked for goodness of fit and considered the final model.

2.4.4 Fungal colonization and GSOB presence

To determine tendencies for trees to have fungal colonization and GSOB presence, Fisher's exact test was used on trees that were intensively sampled. The contingency between presence of each pathogen and symptom or insect damage type (staining, GSOB exit holes, non-GSOB exit holes) or position in tree (crown, the base or above 1 m from the base of the bole) was tested against a null hypothesis of independence for each at $\alpha = 0.05$. For GSOB presence, the contingency table was built using number of GSOB exit holes [scaled at 1–3 (Coleman et al. 2011)] and tree position (on the bole only). When an overall test was significant, then pair-wise differences were determined with Fisher's exact test.

3. Results

A total of 857 trees were mapped and examined for disease and GSOB. Site locations are referred to hereafter by the abbreviations listed in Table 1. A total of 100 fungal species were recovered from 882 samples of symptomatic plant tissues of 428 coast live oak samples collected (Table 2). From all locations, the most frequently recovered pathogens were *D. corticola, F. solani, D. agrifolia,* (aggressive pathogens) and *Do. iberica, Di. verrucaeformis,* and *C. querciphila* (weak pathogens) (Table 2, Fig. 1). Of symptomatic trees, 28% contained an aggressive pathogen, 57% contained a weak pathogen, and 71% contained both aggressive and weak pathogens. *Annulohypoxylon thouarsianum* was present on 38 of 235 trees that exhibited fire scars, on 28 of 194 trees with GSOB, and on eight of 113 trees with western oak bark beetle. No oomycete species were ever recovered from soil or plant tissues. Among all asymptomatic trees sampled, *F. solani* and *C. querciphila* were recovered from one tree each at CNFPC and *Do. iberica* was recovered from one *vs.* three trees at MSC and WGP, respectively.

3.1 Coast live oak mortality

Cumulative coast live oak mortality was not significantly different between sites ($p = 0.0872$), and mortality at WHCP significantly increased from 16.5 to 32.3% ($\chi^2 = 15.2$) between 2010 and 2011 (Fig. 2). The proportion of trees that were dead or nearly dead (Health class = 4) that were infested with GSOB ranged from 33.3 to 54.1% and 55.6 to 100% in 2010 and 2011; for *D. corticola,* the ranges in 2010 and 2011 were 0–53.8% and 0–66.7%, respectively. Cumulative coast live oak mortality was weakly correlated with *D. corticola* and GSOB incidence and negatively correlated with annual relative humidity ($R^2 = 0.4469$, $p < 0.0001$, $p = 0.0003$, and $p = 0.0064$).

3.2 Pathogen and GSOB presence and colonization by host tissues

Multiple logistic regression models were able to explain the presence of individual fungi or GSOB at the tree level (Table 3). Presence of *D. corticola* was negatively correlated with the presence of fire scars ($p = 0.0026$) and woodpecker activity ($p = 0.0058$) and positively correlated with disease abundance ($p = 0.0330$), canker rot ($p = 0.0201$) and symptoms of staining ($p = 0.0020$), non-GSOB exit holes ($p = 0.0111$) and crown symptoms ($p < 0.0001$). Presence of *Do. iberica* was positively correlated with heart rot ($p = 0.0286$) and symptoms of staining ($p = 0.0081$), GSOB ($p = 0.0006$) and non-GSOB exit holes ($p = 0.0043$). Presence of *Di. verrucaeformis* was positively correlated with live tree basal area ($p = 0.0185$) and crown symptoms ($p = 0.0011$). Presence of *F. solani* was positively correlated with fire scars ($p = 0.0132$), disease abundance ($p = 0.0264$), and symptoms of staining ($p < 0.0001$), non-GSOB exit holes ($p = 0.0007$) and crown ($p = 0.0048$). Presence of *C. querciphila* was positively correlated with woodpecker presence ($p < 0.0001$) and staining ($p = 0.0073$) and crown symptoms ($p = 0.0279$). Presence of GSOB was positively correlated with presence of fire scars ($p < 0.0001$) and woodpecker ($p < 0.0001$) and symptoms of staining ($p < 0.0001$) and bark cracking ($p < 0.0001$). Estimates and p values are listed in Table 3.

Apart from a single tissue sample with a trunk symptom at WHCP and CNFPC and eight samples with trunk symptoms at Marian Bear Regional Park (MBRP), all *D. corticola* from GSOB-infested sites was recovered from branches with crown symptoms. Fisher's exact test analysis determined that the presence of *D. corticola, F. solani, Do. iberica, C. querciphila* and *Di. verrucaeformis* was each related to origin of sample location on tree ($p = 0.0194$, 0.0321, 0.0001, <0.0001, <0.0001), and *C. querciphila* was additionally related to symptom type on the bole ($p = 0.0002$) (Figs 3–4). The proportion of samples from which fungi were recovered was significantly greater in the crown than the bole for all fungi except for *F. solani,* which was recovered in greater proportions from the bole. The proportion of *Do. iberica* was greater from upper bole positions, and *D. corticola, F. solani, C. querciphila* and *Di. verrucaeformis* were recovered in equal proportions from both positions of the tree bole (Fig. 3). The proportion of *C. querciphila* recovered was significantly higher at GSOB exit holes than at other bole symptoms (Fig. 4). Association between GSOB severity and bole location could not be detected from the sample data ($p = 0.9734$).

Table 2. Frequency of recovery of fungal species from crown and bole tissue samples collected from coast live oak trees in San Diego and Riverside Counties.

Taxa	Crown	Bole
Amphisphaeriaceae		
Seimatosporium botan		1
Bionectraceae		
Bionectria ochroleuca	9	14
Clonostachys rosea		2
Botryosphaeriaceae		
Botryosphaeria dothidea	1	2
B. fuceliana		1
Camarosporium brabeji	2	1
Diplodia agrifolia	5	2
D. corticola	47	46
D. coryli		1
Discostroma fuscellum	1	
Dothiorella iberica	73	82
Echinodontium taxodium		2
Epicoccum nigrum		2
Fusicoccum quercus		1
Microdiplodia hawaiiensis	1	
Neofusicoccum australe	1	
N. parvum	2	6
Calosphaeriaceae		
Phaeoacremonium aleophilum		2
P. angustius		1
P. inflatipes		1
P. mortoniae	6	6
Ceratobasidiaceae		
Ceratobasidium stevensii		1
Chaetomiaceae		
Chaetomium globosum	2	
Corynesporascaceae		
Corynespora proliferata	3	
Davidiellaceae		
Cladosporium cucumerinum		2
C. cladosporioides		1
Dermateaceae		
Cryptosporiopsis querciphila	74	37
Diaporthaceae		
Phomopsis obscurans		1
Diatrypaceae		
Diatrype stigma	1	3
Diatrypella verrucaeformis	88	12
Eutypa lata	1	
Dothideaceae		
Coniozyma leucospermi	1	1
Helotiales		
Scytalidium lignicola		1
Herpotrichiellaceae		
Phialophora mustea	2	1
Hygromiidae		
Discula quercina	5	4
Hymenochaetaceae		
Inonotus andersonii		1
I. cuticularis	1	1
Hypocreaceae		
Acremonium crotocinigenum		2
Hypocrea koningii		1
H. lixii		2
Trichoderma atroviride	7	5
T. citrinoviride	3	1
T. viride	2	1
Mortierellaceae		
Mortierella elongata	1	1
M. hyalina		1
Mucoraceae		
Absidia glauca		2
Mucor fragilis		1
M. plumbeus	1	4
M. racemosus	1	1

Table 2 Continued

Taxa	Crown	Bole
Nectriaceae		
Cosmospora vilior		1
Fusarium oxysporum		1
F. solani	2	32
Nectria haematococca		3
N. inventa		2
Ophiostomataceae		
Ophiostoma breviusculum	1	1
O. piceae	4	
O. stenoceras	1	1
Peniophoraceae		
Peniophora aurantiaca	1	
Phaeosphaeriaceae		
Sclerostagonospora opuntiae	1	
Plectosphaerellaceae		
Verticillium fungicola		1
Pleosporaceae		
Alternaria alternaria		1
A. citri		1
A. infectoria		2
A. tenuissima	1	
Pleosporales		
Ochrocladosporium elatum		1
Pyrenochaeta cava	1	2
Py. uniguis	1	
Pleurostomataceae		
Pleurostoma ootheca		2
Pleurostomophora richardsiae		3
Psathyrellaceae		
Coprinellus micaceus	1	
Coprinellus radians	1	
Coprinus xantuothlix		1
Schizophyllaceae		
Schizophyllum commune	2	
Ciborinia camelliae	1	
Sordariomycetes		
Eucasphaeria capensis	1	
Stereaceae		
Stereum hirsutum	7	3
Stereum sanguinolentum		3
Trametes hirsuta	5	
Trichocomaceae		
Geosmithia fassatiae		8
G. langdonii		1
G. microcorthyli		1
G. pallida		1
Nigrospora oryzae	1	
Paecilomyces sinensis		1
Pa. variotii		1
Valsaceae		
Cytospora terebinthi		1
C. austromontana	1	4
C. chrysosperma		6
C. eucalypticola		5
C. ribis		4
C. mali		4
C. olrim		1
Vibrisseaceae		
Phialocephala virens		1
Xylariaceae		
Annulohypoxylon thouarsianum	46	45
Biscogniauxia cylindrospora	3	
B. mediterranea	12	6
B. nummularia	2	
B. repanda	3	1
Total	440	413

Names were assigned based on 98–100% homology of the ITS DNA sequences to those in a GenBank BLAST search.

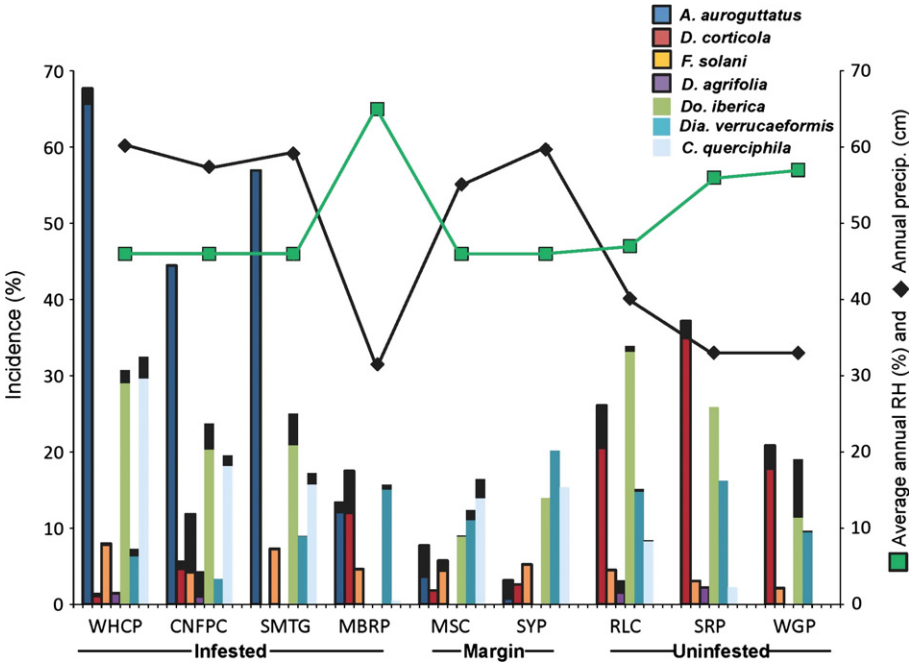

Fig. 1. Annual relative humidity and precipitation, and incidence of goldspotted oak borer and fungal pathogens determined by the proportion of trees per plot with the presence of each agent, averaged for each site in 2010. Incidence values for 2011 are represented in black for each agent.

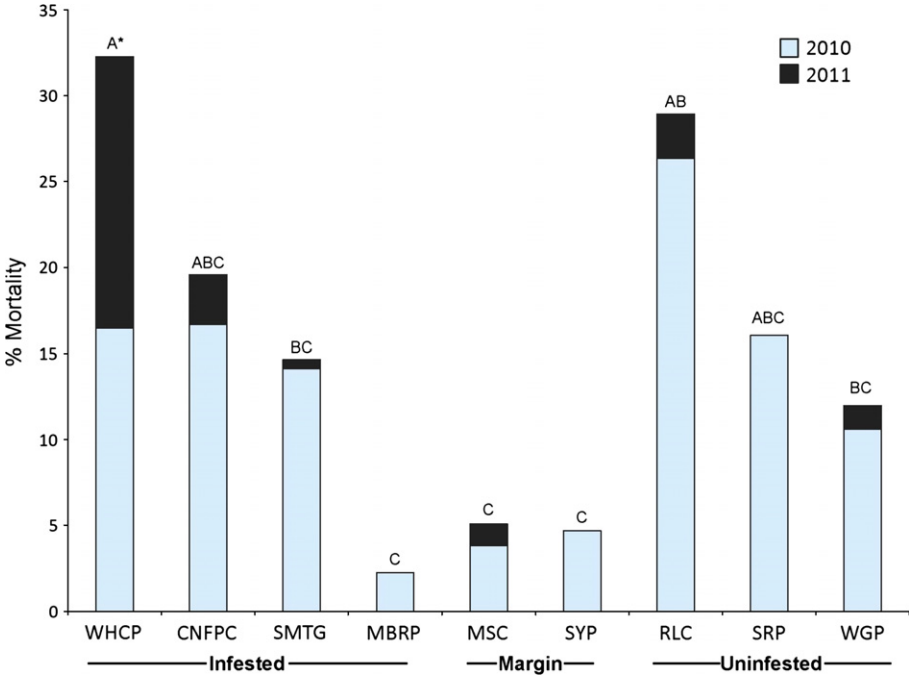

Fig. 2. Average cumulative mortality of *Quercus agrifolia* in 2010 and 2011 by site. Asterisk indicates significant difference between 2010 and 2011 in a chi-squared test (adjusted $\alpha = 0.0056$; critical value = 7.879). Letters indicate differences between sites in 2011.

3.3 Factors associated with plot-level pathogen and GSOB incidence

Multiple linear regression models showed high correlation between environmental variables and plot-level incidence of both GSOB ($R^2 = 0.9183$) and *D. corticola* ($R^2 = 0.7310$) (Table 4). GSOB incidence was positively correlated with annual precipitation ($p = 0.0001$) and annual relative humidity ($p < 0.0001$) and negatively correlated with number of years from

Table 3. Presence of individual pathogens and the goldspotted oak borer (GSOB) at the tree level explained by multiple logistic regression models.

	D. corticola		GSOB		*F. solani*		*Do. iberica*		*Di. verrucaeformis*		*C. querciphila*		*D. agrifolia*	
Variables	Estimate	$p > \chi^2$	Estimate	$p > \chi^2$	Estimate	$p > \chi^2$	Estimate	$p > \chi^2$	Estimate	$p > \chi^2$	Estimate	$p > \chi^2$	Estimate	$p > \chi^2$
Staining	0.4219	0.002	1.2275	<0.0001	0.6734	<0.0001	0.3127	0.0081			0.3296	0.0073	0.769	0.0244
GSOB exit holes							0.4516	0.0006						
Non-GSOB exit holes	0.348	0.0111			0.6445	0.0007	0.3664	0.0043						
Crown Symptoms	0.2832	<0.0001			0.2189	0.0048			0.1734	0.0011	0.1252	0.0279		
Basal area									0.0259	0.0185				
Fire scar[1]	−0.8725	0.0026	1.116	<0.0001	0.5201	0.0132								
Woodpecker	−2.134	0.0058	2.932	<0.0001							1.7171	<0.0001		
Bark cracking			0.5585	<0.0001										
Disease abundance[2]	0.235	0.033			0.3106	0.0264								
Canker rot	1.3228	0.0201												
Heart rot							0.8497	0.0286						

[1]Fire scar categories: 1 = Basal Fire Scar, 2 = Fire Scar on the bole, 3 = Tree Charred.
[2]Disease abundance = Number of fungal diseases present on an individual tree.

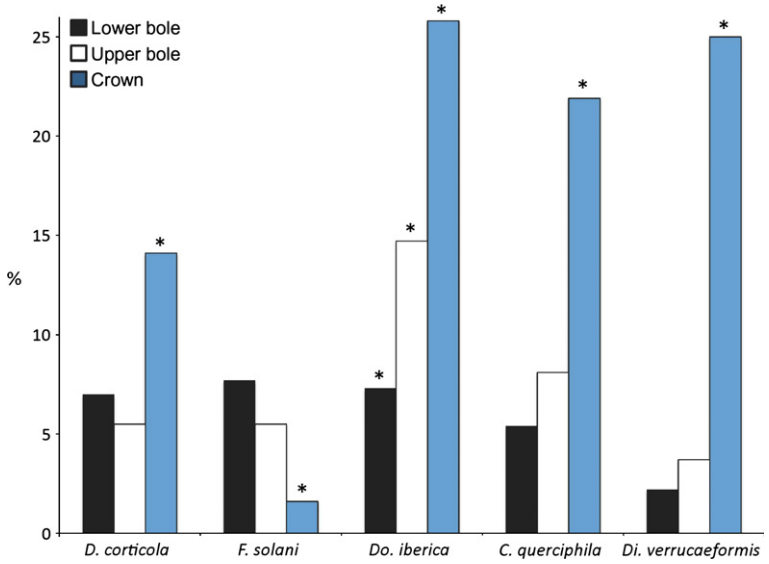

Fig. 3. Recovery of five fungi from crown and bole locations in coast live oak trees. Asterisk indicates significance at $\alpha = 0.05$ by Fischer's exact test.

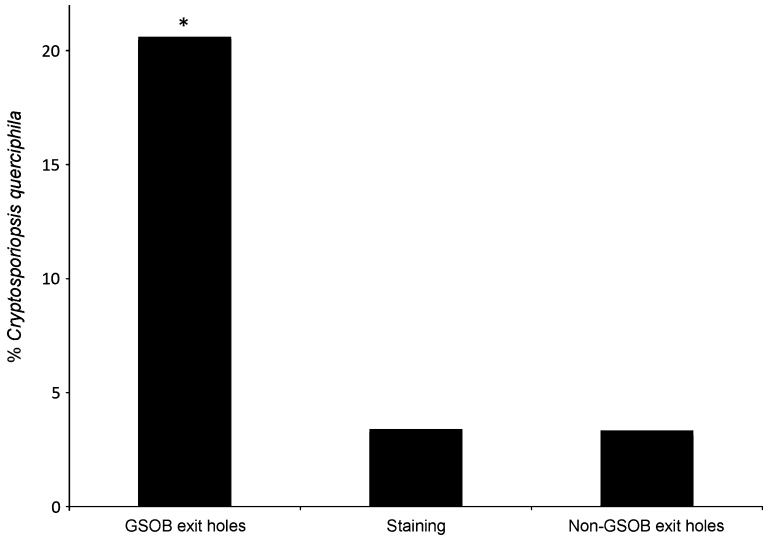

Fig. 4. Differences in recovery of *Cryptosporiopsis querciphila* from symptomatic and insect-damaged tissues of coast live oak. Asterisk indicates significance at $\alpha = 0.05$ by Fischer's exact test.

Table 4. Incidence of *Diplodia corticola* and the goldspotted oak borer (GSOB) at the plot level explained by multiple linear regression models.

	D. corticola[1]			GSOB[2]		
Variables	Estimate	SE	Pr > *F*	Estimate	SE	Pr > *F*
Annual precipitation[3]	−8.561	1.266	<0.0001	23.541	2.724	0.0001
Annual relative humidity[3]	2.252	0.825	0.0094	6.136	0.711	<0.0001
Years since last fire	−0.552	0.151	0.0008	−0.848	0.159	<0.0001
Tree density	−0.036	0.011	0.0019			

[1] $R^2 = 0.7310$.
[2] $R^2 = 0.9183$.
[3] Data obtained from PRISM Climate Group, Oregon State University.

Table 5. Plot-level associations between *D. corticola* and three other agents on *Quercus agrigolia* using the Jaccard index.

Agent	*J*	J_{ran}[1]	SE[2]	*z* Statistic	p-value	+/−Association
GSOB	0.114	0.272	0.055	−2.883	0.004	−
F. solani	0.121	0.261	0.058	−2.416	0.016	−
C. querciphila	0.171	0.324	0.06	−2.57	0.01	−

[1]Computation of *J* from random association of both factors; J_{ran} is critical value for determining positive or negative association.
[2]Standard error.

the most recent fire ($p < 0.0001$). Incidence of *D. corticola* was positively correlated with relative humidity ($p = 0.0094$) and negatively correlated with annual precipitation ($p < 0.0001$), number of years from the previous fire ($p = 0.0008$) and tree density ($p = 0.0019$). Incidence of *D. corticola* was negatively correlated with coast live oak density, but this variable had a 235 times lower contribution than precipitation (-0.036 ± 0.011, $p = 0.0019$).

Disease incidence (DI) for *D. corticola* was highest in GSOB-uninfested locations, with ranges in DI at GSOB-infested, recently infested and uninfested locations in 2010 from 0.0 to 0.12, 0.02 to 0.03 and 0.18 to 0.35, respectively (Fig. 1). DI increased in 2011 by 20.0%, 21.0% and 45.4% at WHCP, CNFPC and MBRP (GSOB-infested), respectively, and 27.0%. 6.4% and 17.0% at RLC, SRP and WGP (GSOB-uninfested), respectively. Yet, mean DI of *D. corticola* at MBRP (0.17 ± 0.03) in 2011 was not significantly different from two of the three GSOB-uninfested sites (Fig. 1, $p = 0.179$ and 0.595, $\alpha = 0.05$). Except for MBRP, the percentage of infected coast live oak was significantly different between GSOB-uninfested sites (0.24 ± 0.04) and other sites (0.05 ± 0.02) ($p = 0.0001$, $\alpha = 0.05$).

Disease incidence (0.06 ± 0.01) was not significantly different between sites for *F. solani* ($p = 0.710$, $\alpha = 0.05$), and increased by 1.7%, 183%, 29.9% and 1.2% at WHCP, CNFPC, (GSOB-infested) MSC (margin) and WGP (GSOB-uninfested) in 2011 (Fig. 1). *D. agrifolia* was present at WHCP and CNFPC and RLC and SRP (GSOB-uninfested), and mean DI was not significantly different between sites (0.03 ± 0.01) ($p = 0.330$, $\alpha = 0.05$), and increased by 7.7%, 308.4% and 100% at WHCP, CNFPC and RLC in 2011 (Fig. 1).

For *Do. iberica*, except for its absence at the recently GSOB-infested MBRP, DI was not significantly different between GSOB-infested and GSOB-uninfested sites (0.26 ± 0.02), but was significantly different between these and recently infested sites (0.11 ± 0.02). In 2011, DI of *Do. iberica* increased by 6.0, 17.0, 1.2, 2.3 and 66.8% at WHCP, CNFPC, MSC, RLC and WGP (Fig. 1). There was no significant difference in DI for *Di. verrucaeformis* between sites (0.12 ± 0.01) ($p = 0.1000$, $\alpha = 0.05$), and DI increased by 15.1%, 3.8%, 11.8%, 2.0% and 1.6% at WHCP, MBRP, MSC, RLC and WGP in 2011. DI for *C. querciphila* was highest at WHCP (0.33 ± 0.09) and lowest at the SRP (0.02 ± 0.02, no change in 2011) ($p = 0.0001$, $\alpha = 0.05$) and absent from WGP. Mean DI for *C. querciphila* at RLC (0.08 ± 0.02) was not different from that of the SRP ($p = 0.308$, $\alpha = 0.05$; Fig. 1). There was no difference in DI of *C. querciphila* at the remaining sites (0.14 ± 0.02).

In 2011, GSOB incidences ranged between 0.44 ± 0.09 and 0.68 ± 0.02 at CNFPC, SMTG and WHCP, and incidences were not significantly different from one another ($p = 0.147$ and 0.0946, $\alpha = 0.05$). Mean GSOB incidence at MBRP was not different from MSC and SYP ($p = 0.4433$ and 0.1689, $\alpha = 0.05$; Fig. 1). Incidence of GSOB increased by 3.0%, 10.3%, 112% and 324% at WHCP, MBRP, MSC and SYP in 2011 (Fig. 1).

3.4 Co-occurrence of agents by site

Most fungal pathogens occupied all GSOB-infested and GSOB-uninfested sites (Fig. 1), with several exceptions: *D. corticola*, *Do. iberica* and *C. querciphila* were each present in all but one site, and the site where they were absent was different for each species (Fig. 1). *Diplodia agrifolia* was present in four of the seven locations, and Jaccard index of association (*J*) showed that *D. corticola* was negatively associated with the presence of GSOB, *F. solani* and *C. querciphila* (values of $Z > 1.96$ and ≤ 1.96 indicate significant positive and negative association with GSOB at $p = 0.05$; Table 5). MBRP (the western-most extent of the GSOB infestation) did not have *Do. iberica* and compared to other GSOB-infested sites had lower incidence of GSOB ($p = 0.0001$, $\alpha = 0.05$) and higher incidence of *D. corticola* ($p = 0.0001$, $\alpha = 0.05$; Fig. 1).

4 Discussion

This study presents a 2-year assessment of the role of pathogens in coast live oak woodlands throughout GSOB-infested and GSOB-uninfested locations in southern California where oak mortality has been observed. Oak decline in California is an example of a complex syndrome involving strong regional differences in factors that are associated with the problem, similar in this respect to oak decline in Europe (Ciesla and Donaubauer 1994; Führer 1998). Given that negligible recovery of pathogens from asymptomatic tissues, confirmation of their pathogenicity in controlled experiments (Lynch et al. 2010, 2013a,b) and that mortality was weakly correlated with RH, *D. corticola* and GSOB incidence, oak decline in southern California appears to be multifaceted, with factors that include climate variables, the effect of GSOB and *D. corticola*, and the added effects of other weak or aggressive pathogens. It should be noted that reported incidence for GSOB is higher than for pathogens because infestation by the beetle was confirmed on dead trees from exit holes, but the presence of pathogens in dead trees is probably underestimated by our reliance on isolation methods that often are only effective on living plant tissues. The set of circumstances for oak decline in southern California is different from that in northern

California, where the decline of oaks and tanoaks is attributed primarily to *Phytophthora ramorum,* causal agent of sudden oak death (Rizzo and Garbelotto 2003).

One reason for the impact of these fungi on coast live oak is not yet understood is because the occurrence and pathogenicity of *D. corticola* and *D. agrifolia* on oaks in the United States have only recently been revealed (Lynch et al. 2010, 2013a). *D. corticola* is an important contributor to cork oak (*Quercus suber*) decline throughout the main cork-producing countries in Europe, including Portugal, Spain, France, Italy and Morocco (Luque et al. 2008), where it also affects *Quercus ilex* and *Quercus cerris* (Alves et al. 2004). *D. mutila*, a close relative of *D. agrifolia* (Lynch et al. 2013a), contributes to decline of *Quercus* spp. throughout Spain (Luque and Girbal 1989; Sánchez et al. 2003). In pathogenicity studies, *D. corticola* was highly aggressive and *D. agrifolia* and *F. solani* were also aggressive (Lynch et al. 2010, 2013a,b).

Periods of severe drought can cause a predisposing stress that in other oaks have manifested in the form of pest and disease attack and mortality up to 10 years after the event (Pedersen 1998). This scenario fits the 'cycle of decline' paradigm for forest trees that is characterized by multiple pathogens and insect agents interacting over time (Manion and Lachance 1992) and is a very different problem from diseases that have only one fungal species operating individually to cause epidemics. Although southern California has experienced periods of severe drought, Mahall et al. (2009) showed that coast live oak trees may be adapted to escape detrimental effects of summer drought by having extensive and deep root systems that tap into supplies of perennially available groundwater when comparing water relations, chlorophyll fluorescence and leaf gas exchange between coast live oak adult trees and seedlings in the field. Based on the root architecture of coast live oak (Cannon 1914; Callaway 1990), escape of drought stress is highly plausible. Furthermore, results from measuring water potential on coast live oak trees either attacked or not attacked by GSOB suggest that trees prone to attack by GSOB were not drought stressed (Coleman et al. 2011).

The ability of *D. corticola*, *D. agrifolia, F. solani* (Lynch et al. 2013a,b) and GSOB (Coleman et al. 2011) to attack healthy plants that are not predisposed to stress indicates that each of these agents are factors capable of inciting decline on individual trees. A greater incidence of *D. corticola* and GSOB suggests these agents are more significant causal agents of decline over a landscape, while agents such as *Do. iberica, C. querciphila* and *Di. verrucaeformis* weakly colonized healthy seedlings (Lynch et al. 2013a,b), negligible recovery of these agents from asymptomatic tissues suggests they are factors contributing to decline in trees already exposed to other stress factors (Sinclair 1965; Manion 1981). It has been shown that GSOB potentially increases drought stress in trees (Coleman et al. 2011). Yet, it may be that a seemingly weak agent such as *C. querciphila,* which is prevalent throughout GSOB-infested sites and has the strongest association to symptoms of GSOB, may contribute to the furthering of crown dieback on a tree. It is likely that *Di. verrucaeformis* is an underlying cause of background irritation or low-level stress to the trees, given that incidence of this agent was not significantly different between sites. The fact that *Do. iberica* is found in association with all types of symptoms and was significantly different between locations with high *vs.* relatively lower mortality suggests its role as an opportunistic pathogen that further alters the health of adult trees.

Although multiple agents occupy the same habitat, several lines of evidence indicate that *D. corticola* and GSOB are mortality agents that can act independently of each other. Low Jaccard index values from association analysis of pairs of agents indicate lower than random co-occurrence in trees. In the current study, these indicate a much lower chance that *D. corticola* co-occurs with *F. solani*, *C. querciphila* and GSOB organisms. Sites with GSOB would more likely have *F. solani* and *C. querciphila*. In addition, incidence of *D. corticola* was low overall throughout GSOB-infested locations but high in GSOB-uninfested sites, and *D. corticola* was recovered from symptomatic tissues within GSOB-infested sites on portions of trees away from GSOB attack (i.e. cankered branches *vs.* bole). Finally, annual precipitation was a factor indicated as significant in multiple linear regression analyses for both GSOB and *D. corticola* incidences, but with differing correlations. GSOB-infested sites generally experience higher annual precipitation, suggesting that precipitation drives the establishment and persistence of a dominant agent (either GSOB or *D. corticola*). MBRP is a unique site from which no *Do. iberica* was recovered and showed moderate levels of *D. cortiola* and GSOB incidences. Increased understanding of how decline will operate at this site will require further monitoring.

Goldspotted oak borer-infested sites generally occurred in areas with historically higher annual precipitation. Interpretation of the significance of this correlation is difficult. Three conflicting explanations are (i) it is possible that higher precipitation could be involved in establishment and persistence of a dominant agent (either GSOB or *D. corticola*); in this case, the mechanisms by which these agents cause decline on individual trees could still differ, which may then show differences in decline over a landscape; (ii) it is also possible that correlations between GSOB and perhaps one or more of the pathogens with precipitation could be an artefact of an expansion of an agent from a site of introduction; in this case, subsequent expansion into broader environments may change its correlations with environmental factors; (iii) it is possible that there is an undetected relationship between areas of high historic precipitation and the effects of recent drought, so that deviations from normal precipitation may be the true factor that predisposes trees to one or another agent of decline. Further studies using multiple genetic markers may reveal which of the pathogens, if any, have low genetic diversity and were recently introduced, versus a more diverse genetic signature more usual of endemic pathogens.

Even in areas where GSOB is the primary agent of decline of mature trees, *D. corticola* may have an effect on recruitment of new trees into the system because this species can kill seedlings (Lynch et al. 2013a). This possibility is of especial concern given that recruitment of oaks in savannas and woodlands appears to be substantially declining throughout the northern hemisphere (Shaw 1968; Loftis and McGee 1993; Palmer et al. 2004; Tyler et al. 2006). For coast live oak in particular, sites with herbaceous understories that are not shrub-dominated tend to be associated with declining populations (Callaway and Davis 1998) as shrubs can have strong facilitative effects on the survival of *Q. agrifolia* seedlings (Callaway and D'Antonio 1991). With the added challenge of an aggressive pathogen like *D. corticola* threatening the survival of

seedlings, regeneration success within GSOB-infested sites that are deprived of a shrubby understory could be especially difficult; this possibility would need to be investigated further.

On an individual tree, the presence of a pathogen may be explained by a combination of variables, which include symptoms, tree location and other abiotic and biotic factors. Many disease agents (not including *Di. verrucaeformis*) were associated with trunk staining, thus other attributes on a tree must be considered while determining the likelihood of the presence of a particular agent. Exit holes caused by other wood-boring insects (usually the flat-headed apple borer or the western sycamore borer) could be serving as infection courts for *D. corticola*, *F. solani* and *Do. iberica*. *D. corticola* and *F. solani* were also positively correlated with crown symptoms, but the latter is most often found on the bole of the tree. *Do. iberica* is more often found in the crown and may also be found in association with GSOB exit holes, again suggesting its role as an opportunistic pathogen. Taking into account the conditions of a tree in total, a tree that has only staining, non-GSOB exit holes, crown symptoms and canker rot would be most likely to have *D. corticola,* whereas a tree with fire scars would more likely have *F. solani*. A tree with heart rot would most likely have *Do. iberica* as opposed to *F. solani*, with the presence of fire scars not being an important factor for *Do. iberica*. If there is no canker rot and heart rot, a tree with signs of woodpecker activity, staining and crown symptoms will most likely have *C. querciphila*. A tree with the combination of bark cracking, woodpecker activity, staining and fire scars is most likely a GSOB tree. Most likely, staining symptoms associated with GSOB activity would yield recovery of *C. querciphila* from the bole.

Given that *D. agrifolia* was recovered at low incidence levels and is a newly described species (Lynch et al. 2013a), it is likely that this species is new to California. Although not as aggressive as *D. corticola* (Lynch et al. 2013a), the impact it may have within these forests in combination could be significant. Incidence of *D. agrifolia* as well as *F. solani* increased within one year, suggesting that we may expect detection of these agents from symptomatic tissues to increase; despite the fact that levels are currently low, they could potentially have more of a presence and have more impact over the long term.

An additional area that requires further investigation is the role of bacterial pathogens interacting with fungi and insects in the decline as seen with European oaks (Biosca et al. 2003; Denman and Webber 2009; Denman 2010). In addition to two isolates of *Gibbsiella quercinecans* (Brady et al. 2010) (first record), several unknown or new species of *Brennaria* were isolated from stains on a single black oak tree (*Q. kelloggii*) in a GSOB-infested site (Denman 2010). These bacteria have also been recovered from several coast live oak trees throughout sites of the present study (S. C. Lynch and A. Eskalen, unpublished data) and have been found in association with acute oak decline of *Q. robur* and *Q. petraea* in Britain (Denman 2010). Larvae of *Agrilus biguttatus* have been seen in close proximity to necrotic areas of bleeding trunk cankers, but this relationship is not always present and is little understood (Denman 2010). Species identification, pathogenicity, distribution, and interactions with GSOB and fungi from the present study need to be examined to clarify the role of pathogenic bacteria more fully.

With the recent emergence of these agents only beginning to be understood, causes of decline and alteration of stand dynamics on southern California oak woodlands will need to be studied over the long term to understand the mechanism of oak decline by which and to what degree a complex of agents alter oak woodland processes throughout southern California.

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