3. PUBLIC FORUM

Today's Item **Information** ☒ **Action** ☐

Receipt of public comments and requests for regulatory and non-regulatory actions.

Summary of Previous/Future Actions

- **Today's receipt of requests and comments** **Dec 9-10, 2015; San Diego**
- Direction to grant, deny, or refer requests Feb 10-11, 2016; Sacramento

Background

FGC generally receives three types of correspondence: Requests for regulatory action, requests for non-regulatory action, and informational only. The Administrative Procedure Act (APA) requires action on regulatory requests to be either denied or granted and notice made of that determination. At the end of public forum a motion may be made to provide direction to staff on any items for which FGC wishes to receive additional information or take immediate action. Otherwise, FGC will determine the fate of the regulatory and non-regulatory requests at the next commission meeting to allow staff time to evaluate requests.

Significant Public Comments

1. See regulatory requests in Exhibit 1
2. See non-regulatory requests in Exhibit 2

Recommendation (N/A)

Exhibits

1. Table containing a summary of new petitions for regulation change received by Nov 24 at 5:00 p.m., the comment deadline for the meeting binder.
2. Table containing a summary of new non-regulatory requests received by Nov 24 at 5:00 p.m., the comment deadline for the meeting binder.

3-9. Individual, new petitions and requests that are summarized in the tables.

10-17. Informational-only items; staff will not take any action on these unless otherwise directed by FGC.

Motion/Direction (N/A)

CALIFORNIA FISH AND GAME COMMISSION
REQUESTS FOR REGULATORY ACTION THROUGH 11-24-2015

FGC - California Fish and Game Commission **DFW** - California Department of Fish and Wildlife **WRC** - Wildlife Resources Committee **MRC** - Marine Resources Committee

Grant (previously Accept): FGC is *willing to consider* the petition through a process
Refer: FGC *needs more information* before deciding whether to grant or deny the petition
Deny (previously Reject): FGC is *not willing to consider* the petition

Green cells: Referrals to DFW for more information
Lavender cells: Accepted and moved to a rulemaking
Blue cells: Referrals to FGC staff or committee for more information
Yellow cells: Current action items

Tracking No.	Date Received	Date Response Due (10 woking days)	Response letter to Petitioner	Accept or Reject	Name of Petitioner	Subject of Request	Code or Title 14 Section Number	Short Description	FGC Decision
2015-003	10/27/2015	11/10/15	11/10/2015	A	Eric Boyd	Allow 50 Shot Shells	Sections 551 and 552, Title 14, CCR	Allow hunters using a state wildlife area or national wildlife area refuge to carry 50 shot shells in field. Currently, hunters are allowed 25 shot shells.	**Action scheduled 2/10-11/2016**
N/A	2/12/2015	N/A	N/A	N/A	Jim Cloninger	Deer Hunting Tags		Requests changes to deer, elk, pronghorn, and sheep hunting tag draw to reduce cost and be more competitive with border states.	**Action scheduled 2/10-11/2016**
2015-004	11/2/2015	11/16/15	11/16/2015	A	James McCabe	Native Reptiles Venom	Sections 5061	Commerical take, sale, trasnport, export or import of native reptiles	**Action scheduled 2/10-11/2016**
2015-005	11/12/2015	11/25/15	11/16/2015	R	Corey Nommensen	Domesticated Ferrets	Under 671 14CA ADC 671	Domesticated ferrets are misclassified as non-domestic and subject ot ban under 671	**Action scheduled 2/10-11/2016**
2015-006	11/24/2015	12/10/15	11/25/2015	A	Dennis Thibeault	Rockport Rocks	632(b)(17)	Remove special closure regulations for Rockport Rocks.	**Action scheduled 2/10-11/2016**

CALIFORNIA FISH AND GAME COMMISSION
REQUESTS FOR NON-REGULATORY ACTION THROUGH 11-24-2015

FGC - California Fish and Game Commission **DFW** - California Department of Fish and Wildlife **WRC** - Wildlife Resources Committee **MRC** - Marine Resources Committee

Grant (previously Accept): FGC is *willing to consider* the petition through a process **Deny (previously Reject):** FGC is *not willing to consider* the petition
Refer: FGC *needs more information* before deciding whether to grant or deny the petition

Green cells: Referrals to DFW for more information **Blue cells:** Referrals to FGC staff or committee for more information
Lavender cells: Accepted and moved to a rulemaking **Yellow cells:** Current action items

Date Received	Name of Petitioner	Subject of Request	Short Description	FGC Decision	DFW/FGC Staff Response	Final Action, Other Outcomes
11/1/2015	Kerry Kriger	Bullfrog policy	Requests response to letter sent to Director Bonham and Secretary Laird concerning status of bullfrog vision document and discontinuing importation permits.	**Action Scheduled 2/10-11/2016**	Deny - not FGC authority	
11/19/2015	Marc Gorelnik	D-Crab Closure	Requests December meeting agenda include a specific item to discuss the ongoing closures of the recreational Dungeness and rock crab fisheries.	**Action Scheduled 2/10-11/2016**	Grant - will be discussed at Dec FGC meeting	

From: Jim Cloninger
To: FGC
Subject: Proposal: How to obtain a deer hunting tag in California
Date: Thursday, February 12, 2015 11:13:59 AM

Fish and Game Commission: I want to propose the following procedure to obtain a resident deer hunting tag in California.

Proposal: Conduct the deer hunting draw for a deer tag the same as the draw for elk, pronghorn and sheep. Namely, resident applicant pays an application fee, the amount the same as the elk, pronghorn and sheep application fee. The actual draw will be the same. A deer tag applicant would pay the application fee, submit an application with up to 3 deer hunting zones. If the applicant is successful for any of his/her zone choices, he/she is entitled to purchase a deer hunting tag for that zone. If the applicant is not successful in drawing his/her first choice, a preference point will be granted. If the applicant is not successful for any of his/her choices, he/she may purchase a "left over" deer hunting tag, or not hunt deer that season.

Discussion: I have conducted a survey of the states that border California to determine a procedure norm for a resident to obtain a deer hunting tag.

Oregon: Must purchase a hunting license - $29.50. Application fee to apply for a deer hunting tag - $8.00. If successful in the draw, deer hunting tag - $24.50.

Nevada: Must purchase a hunting license - $33.00. Application fee to apply for a deer hunting tag - $13.00. If successful in the draw, deer hunting tag - $30.00.

Arizona: Must purchase a hunting license - $37.00. Application fee to apply for a deer hunting tag - $13.00. If successful in the draw, deer hunting tag - $45.00.

California: Must purchase a hunting license - $46.40. Must purchase a non refundable deer hunting tag before the draw - $30.81.

As you can see an applicant for a deer hunting tag in California is more expensive: $30.81 for California, $13.00 for Nevada, $13.00 for Arizona, $8.00 for Oregon. The average application fee for obtaining a deer hunting tag in bordering states is $11.33. A California applicant has to pay $19.48 more for being unsuccessful in the deer hunting tag draw!!!! California's procedure must be changed to the normal procedure as used in bordering states.

James Cloninger, Antioch,CA
[redacted]

Tracking Number: (BCD-001)

To request a change to regulations under the authority of the California Fish and Game Commission (Commission), you are required to submit this completed form to: California Fish and Game Commission, 1416 Ninth Street, Suite 1320, Sacramento, CA 95814 or via email to FGC@fgc.ca.gov. Note: This form is not intended for listing petitions for threatened or endangered species (see Section 670.1 of Title 14).

Incomplete forms will not be accepted. A petition is incomplete if it is not submitted on this form or fails to contain necessary information in each of the required categories listed on this form (Section I). A petition will be rejected if it does not pertain to issues under the Commission's authority. A petition may be denied if any petition requesting a functionally equivalent regulation change was considered within the previous 12 months and no information or data is being submitted beyond what was previously submitted. If you need help with this form, please contact Commission staff at (916) 653-4899 or FGC@fgc.ca.gov.

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CALIFORNIA
FISH AND GAME
COMMISSION
2015 SEP 29 PM 2:05
MLS

SECTION I: Required Information.

Please be succinct. Responses for Section I should not exceed five pages

1. **Person or organization requesting the change (Required)**
   Name of primary contact person: Curtis Haney
   Address: [redacted]
   Telephone number: [redacted]
   Email address: [redacted]

2. **Rulemaking Authority (Required)** - Reference to the statutory or constitutional authority of the Commission to take the action requested: Title 14, CONS, FGC, Division 1, Chapter 2, Article1, Section 205

3. **Overview (Required)** - Summarize the proposed changes to regulations: Change CA F&W Regulations, Chapter 4, (Ocean Fishing), Section 29.05 (b) (1) (Invertebrates, General) (Add Barnacles to the list of Invertebrates that can be harvested, and remove them from being prohibited to, "Take or Possess").

4. **Rationale (Required)** - Describe the problem and the reason for the proposed change: "Take or Possession" of barnacles in California ocean waters is currently prohibited. I and many of my fellow divers love the taste of Gooseneck/Leaf Barnacles which are abundant on rocks exposed at low tide. Currently, the only legal way to consume barnacles locally is to go to an expensive restaurant and order them off the menu. Barnacles served in restaurants are all imported from Spain. I personally spoke with Jerry Kashiwada, a marine biologist at the CDFW office in Fort Bragg and asked him why Barnacles could not be harvested. The only reason he could think of was that when the regulations were written it was assumed that no one harvested or ate barnacles! He suggested I petition the commission to have barnacles added to the list of invertebrates that are allowed to be harvested.

SECTION II: Optional Information

5. **Date of Petition: 26 September 2015**

6. **Category of Proposed Change**
   ☒ Sport Fishing
   ☐ Commercial Fishing
   ☐ Hunting
   ☐ Other, please specify: Click here to enter text.

7. **The proposal is to:** (*To determine section number(s), see current year regulation booklet or https://govt.westlaw.com/caireqs*)
   ☐ Amend Title 14 Section(s):Chapter 4, Section 29.05 (b) (1)
   ☐ Add New Title 14 Section(s): NA
   ☐ Repeal Title 14 Section(s): NA

8. **If the proposal is related to a previously submitted petition that was rejected, specify the tracking number of the previously submitted petition** Click here to enter text.
   Or ☒ Not applicable.

9. **Effective date**: If applicable, identify the desired effective date of the regulation.
   If the proposed change requires immediate implementation, explain the nature of the emergency: 2017 - 2018 Regulation Booklet Update

10. **Supporting documentation:** Identify and attach to the petition any information supporting the proposal including data, reports and other documents: None

11. **Economic or Fiscal Impacts:** Identify any known impacts of the proposed regulation change on revenues to the California Department of Fish and Wildlife, individuals, businesses, jobs, other state agencies, local agencies, schools, or housing: None

12. **Forms:** If applicable, list any forms to be created, amended or repealed:
    None

SECTION 3: FGC Staff Only

Date received: Click here to enter text.

FGC staff action:
☐ Accept - complete
☐ Reject - incomplete
☐ Reject - outside scope of FGC authority

Tracking Number

Date petitioner was notified of receipt of petition and pending action: _____

Meeting date for FGC consideration: _____

FGC action:
☐ Denied by FGC

☐ Denied - same as petition _____
Tracking Number

☐ Granted for consideration of regulation change

Tracking Number: 2015-003 (Click here to enter text.)

To request a change to regulations under the authority of the California Fish and Game Commission (Commission), you are required to submit this completed form to: California Fish and Game Commission, 1416 Ninth Street, Suite 1320, Sacramento, CA 95814 or via email to FGC@fgc.ca.gov. Note: This form is not intended for listing petitions for threatened or endangered species (see Section 670.1 of Title 14).

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SECTION I: Required Information.

Please be succinct. Responses for Section I should not exceed five pages

1. **Person or organization requesting the change (Required)**
   Name of primary contact person: Eric Boyd
   Address: [redacted]
   Telephone number: [redacted]
   Email address: [redacted]

2. **Rulemaking Authority (Required)** - Reference to the statutory or constitutional authority of the Commission to take the action requested: Title 14, Division 1, Subdivision 2, Chapter 8, Sections 551 and 552

3. **Overview (Required)** - Summarize the proposed changes to regulations: Allow hunters using a State of California Wildlife area or National Wildlife refuge to carry 50 shot shells in the field. [Currently hunters are allowed only 25 shot shells].

4. **Rationale (Required)** - Describe the problem and the reason for the proposed change: Although the bag limits for geese have risen dramatically over the last several years, hunters are still crippled by the 25 shot shell limit on Wildlife Areas and NWR's. The goose limits alone are now 25 birds. Coupled with the possibility of 7 ducks as well and the 25 shot limit has become a rule preventing anyone from actually harvesting a full limit of ducks and geese. I have tried to work with the Department of Fish and Wildlife over the last several years on this change. I have been unsuccessful. It is now necessary to bring this directly to the Commission. Please change this rule to a shell limit that makes sense. A maximum of 50 shells would be logical.

SECTION II: Optional Information

5. **Date of Petition: 10/202015**

6. **Category of Proposed Change**

☐ Sport Fishing
☐ Commercial Fishing
☒ Hunting
☐ Other, please specify: Click here to enter text.

7. **The proposal is to:** (*To determine section number(s), see current year regulation booklet or https://govt.westlaw.com/calregs*)
☒ Amend Title 14 Section(s):551 and 552
☐ Add New Title 14 Section(s): Click here to enter text.
☐ Repeal Title 14 Section(s): Click here to enter text.

8. **If the proposal is related to a previously submitted petition that was rejected, specify the tracking number of the previously submitted petition** Click here to enter text.
Or ☒ Not applicable.

9. **Effective date**: If applicable, identify the desired effective date of the regulation.
If the proposed change requires immediate implementation, explain the nature of the emergency: December 1, 2015

10. **Supporting documentation:** Identify and attach to the petition any information supporting the proposal including data, reports and other documents: see enclosed information regarding effective shot sizes for ducks versus geese.

11. **Economic or Fiscal Impacts:** Identify any known impacts of the proposed regulation change on revenues to the California Department of Fish and Wildlife, individuals, businesses, jobs, other state agencies, local agencies, schools, or housing: none

12. **Forms:** If applicable, list any forms to be created, amended or repealed:
none

SECTION 3: FGC Staff Only

Date received: Click here to enter text. 10/27/15

FGC staff action:
☒ Accept - complete
☐ Reject - incomplete
☐ Reject - outside scope of FGC authority

Tracking Number

Date petitioner was notified of receipt of petition and pending action: 11/10/15

Meeting date for FGC consideration: Dec 2015 / Feb 2016

FGC action:
☐ Denied by FGC
☐ Denied - same as petition _____
Tracking Number

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2015 OCT 27 AM 8:42

☐ Granted for consideration of regulation change

10/20/2015

General survey and information regarding shot size effectiveness on ducks and geese.

Since non-toxic shot has been required to hunt waterfowl in the USA there have been many studies and tests performed by various researchers and ballistics experts on effectiveness of shot sizes to take ducks and geese. These groups and individuals include:

Ducks Unlimited
L. P. Brezny. Gun Digest Guide to Modern Shotgunning
Tom Roster, Shotshell Ballistics Editor, American Waterfowler magazine
See included T. Roster Lethality Table referenced by the Wisconsin Department of Natural Resources: **dnr.wi.gov/topic/hunt/documents/tomrosternontoxicshot.pdf - 19k - 2013-02-18**
California Waterfowl Association

Mr. Roster's data attached show the best selection for geese is steel shot size BBB and for ducks size #3's.

Therefore in order to maximize cleanly taking geese or ducks and minimize crippling you should be shooting two different shot sizes. Since the restriction for shot shells is now 25 total, hunters typically carry only the shot size for birds they expect to find the most opportunities in public areas. Unfortunately if they carry shot for ducks but have opportunities for geese, they will attempt to take the larger birds with the small shot size. This results in many crippled and lost birds due to the less effective shot size. The opposite is also true, that if the hunter is shooting the larger shot for geese, if taking ducks is attempted, the larger shot with more open patterns and fewer pellets per shell will cause more crippling of ducks.

With the opportunity to take 25 geese and 7 ducks in the field, it is logical and prudent to allow for the proper amount and shot size for these two different species. A 50 shot shell limit would allow hunters to carry the correct shot sizes for the given opportunities. Maintaining a 25 shell limit is neither logical from the standpoint of daily bag limits nor conducive to proper conservation and clean harvest of waterfowl.

Eric Boyd

Eric Boyd

©TOM ROSTER'S 2013 NONTOXIC SHOT LETHALITY TABLE©

Proven Nontoxic Shot Loads For Waterfowl & Upland Game Birds[1] *Load Velocity: 1,225 - 1,600 FPS* ACTIVITY	Typical Shooting Range of Activity (Yards)	Most Effective Nontoxic Shot Size(s) For Birds Listed Under ACTIVITY At The Distances Listed In The Second Column	Minimum Load Weight (Ounces)	Minimum Pellet Hits Needed on Lethal Areas for Clean Kills	Minimum Pattern Count Needed at Any Distance for Clean Kills (# of Pellets in 30" Circle)	Most Effective Choke(s) (Given in Lead Shot Choke Designations)
Large Geese At Long Range	50-65	Steel BBB to T	1-1/4	1-2	50-55	Improved Modified
Giant, Western, Atlantic and Interior Canadas	50-70	HEVI-Shot 2 to B	1-1/2	1-2	50-55	Improved Modified, Full
Large Geese Over Decoys	35-50	Steel BB to BBB	1-1/4	1-2	50-55	Improved Cylinder, Modified
	35-50	HEVI-Shot 2 to B	1-1/2	1-2	50-55	Improved Cylinder, Modified
Medium/Small Geese Long Range	50-65	Steel BB to BBB	1-1/4	1-2	60-65	Improved Modified
Snow, White-fronted, Lesser Canadas	50-65	HEVI-Shot 2	1-1/2	1-2	60-65	Improved Modified, Full
Medium/Small Geese Over Decoys	35-50	Steel 2 to BB	1-1/8	1-2	60-65	Light Modified, Modified
	35-50	HEVI-Shot 4 to 2	1-1/4	1-2	60-65	Improved Cylinder, Modified
Large Ducks At Long Range	45-65	Steel 2 to 1	1-1/8	1-2	85-90	Improved Modified, Full
Mallard, Black, Pintail, Goldeneye, Gadwall	45-65	HEVI-Shot 4	1-1/4	1-2	85-90	Improved Modified, Full
Large Ducks Over Decoys	20-45	Steel 6 to 2	¾ - 1	1-2	85-90	I.C. (20-35 Yds), Mod. (35-45 Yds)
	20-45	HEVI-Shot 6 to 4	1-1/8	1-2	85-90	I.C. (20-35 Yds), Mod. (35-45 Yds)
Medium Ducks Over Decoys	20-45	Steel 6 to 3	1	1-2	115-120	I.C. (20-35 Yds), Mod. (35-45 Yds)
Wigeon, Scaup, Shoveler	20-45	HEVI-Shot 6 to 4	1-1/8	1-2	115-120	I.C. (20-35 Yds), Mod. (35-45 Yds)
Small Ducks Over Decoys	20-45	Steel 6 to 4	1	1-2	135-145	Mod. (20-35 Yds), Full (35-45 Yds)
Teal, Ruddy, Bufflehead	20-45	HEVI-Shot 6	1-1/8	1-2	135-145	Mod. (20-35 Yds), Full (35-45 Yds)
Ring-Necked Pheasants	20-50	Steel 3 to 2	1	2-3	90-95	I.C. (20-30 Yds), Mod. (30-50 Yds)
	20-50	HEVI-Shot 6 to 4	1-1/8	2-3	90-95	I.C. (20-30 Yds), Mod. (30-50 Yds)
Turkeys (Head and Neck Shots)	20-40	Steel 4	1-1/4	3-4	210-230	Full or Extra Full
Northern Bobwhite Quail	20-30	Steel 7	5/8	1-2	170-190	Imp. Cyl., Light Modified
Swatter Load For Wounded Birds	20-30	Steel 7 to 6	1	1	175	Improved Modified, Full

NOTE: The pellets in the steel shot loads listed in this table were traditional, highly spherical ball-shaped pellets of ~ 7.86 g/cc density and 90-95 DPH hardness. The HEVI-Shot pellets were of 12.0 g/cc density and slightly harder than traditional steel pellets.

This table summarizes Tom Roster's analyses to date of the waterfowl lethality data bases for certain of the 15 U.S. steel versus lead waterfowl shooting tests run between 1968 & 1982 & one steel-only pheasant shooting test plus lethality data bases owned by ammunition companies for birds taken with nontoxic shotshell loads Roster tested for them & the CONSEP organization.

Note: Steel #BBB (.190") and HEVI-Shot #2 (.150") have exhibited the best all-around performance for taking geese; steel #3 (.140") and HEVI-Shot #4 (.130") the best all-around performance for taking ducks; and steel #2 and HEVI-Shot #4 the best all-around performance for taking ring-necked pheasants.

[1]These findings are derived from testing 3" 20 gauge; 2¾", 3" and 3½" 12 gauge; and 3½" 10 gauge steel loads; plus 3" 20 gauge and 2¾" and 3" 12 gauge HEVI-Shot loads.

© Copyright 2013 by Tom Roster. For answers to questions on this table contact: Tom Roster, 1190 Lynnewood, Klamath Falls, OR, USA 97601. tomroster@charter.net

Tracking Number: (2015_004)

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SECTION I: Required Information.

Please be succinct. Responses for Section I should not exceed five pages

1. Person or organization requesting the change (Required)
Name of primary contact person: James G. McCabe
Address: [redacted]
Telephone number: [redacted]
Email address: [redacted]

2. Rulemaking Authority (Required) - Reference to the statutory or constitutional authority of the Commission to take the action requested: The Fish and Game Code, Section: §5061 Rules and Regulations. Authorizes the Fish and Game Commission to establish regulations for commercial take, sale, transport, export or import of native reptiles. To date, the Commission has adopted two regulations under this authority. The first regulation is: CCR, Title 14, Section 651, Commercial Take of Native Reptiles and Amphibians for Scientific or Educational Institutions. The second regulation is: CCR, Title 14, Section 43, Captive Propagation and Commercialization of Native Reptiles. We are requesting the Commission to modify existing regulations or establish new regulations that would permit the collection of venom from native reptiles for the commercial production of vaccines, anti-venom and other therapeutics agents; for both domesticated animals and human use. The purpose is to produce effective products against the bites from native venomous snakes. CCR. Title 14, Section 43 pertains to the production of captive born reptiles for the purpose of selling them in the pet trade and has no application to the commercialization of venom or products produced from venom. CCR, Title 14, Section 651, Commercial Take of Native Reptiles and Amphibians for Scientific or Educational Institutions could be modified to accommodate the activities we are describing. We would need the Commission to expand the definition of scientific and educational organization to include commercial operations which would be permitted to maintain venomous reptiles for the purpose of venom extraction for the production of vaccines, antivenom and other therapeutic agents. §40. General Provisions Relating to Native Reptiles and Amphibians; (f) Biological Supply Houses and Exempt Organizations is another section that could be modified or additions made to the code to accommodate the commercialization of venom. These options are discussed in detail below in section 3. Overview.

3. Overview (Required) - Summarize the proposed changes to regulations: The Fish and Game Code, Section: 5061 authorizes the Fish and Game Commission to establish regulations for commercial take, sale, transport, export or import of native reptiles. We would like the Commission to create new regulations which would include the following points: **1.** Allow for the venom from native reptiles to be collected, processed and stored for commercial use to produce vaccines, antivenom and other therapeutic products. **2.** The new regulation(s) would make provisions for trained, experienced personnel to be permitted to hold and maintain native venomous reptiles under humane conditions for prolonged periods of time to allow for multiple venom extractions. **3.** Venomous reptiles maintained for the purpose of venom extraction will be exempt from bag limits as they will not be considered under the "sport take" provisions of the Fish and Wildlife regulations. **4.** Venomous reptiles used for the purpose of venom extraction would be classified as nuisance animals that would otherwise be destroyed as they pose a threat to domestic animals or human life. **5.** Individuals or Companies permitted to commercialize the venom from native reptiles will **not** be allowed to purchase native venomous reptiles or the venom from native reptiles in any form (i.e.: Lyophilized or "raw" unprocessed liquid) from a third party source. This is to discourage the commercial hunting of native snakes. CCR, Title 14, Section 651, Commercial Take of Native Reptiles and Amphibians for Scientific or Educational Institutions could be modified to accommodate the activities we are describing. We would need the Commission to expand the definition of "Organization" to include commercial operations which produce vaccines, antivenom and other therapeutic agents. §40. General Provisions Relating to Native Reptiles and Amphibians; (f) Biological Supply Houses and Exempt Organizations is another section that could be modified or additions made to the code to accommodate the commercialization of venom. One possible modification to section (f), (2) Organizations and Schools Exempt from Permit; could read: "Organizations that extract venom from native reptiles for biomedical research or therapeutic products are exempt from permit. Another possible option would be to create a paragraph (f), ("3") that could state: The Department may issue permits to owners of businesses that extract venom from reptiles for the production of Therapeutic/Biomedical products and/or biomedical research.

4. Rationale (Required) - Describe the problem and the reason for the proposed change: Our published research on the efficacy of the only commercially available canine rattlesnake vaccine lead us to the conclusion that the this vaccine is ineffective against the bite from the two most commonly encountered rattlesnakes in California, the Northern and Southern Pacific Rattlesnakes [Comparison of the protective effect of a commercially available western diamondback rattlesnake toxoid vaccine for dogs against envenomation of mice with western diamondback rattlesnake (Crotalus atrox), northern Pacific rattlesnake (Crotalus oreganus oreganus), and southern Pacific rattlesnake (Crotalus oreganus helleri) venom., Am J Vet Res. 2015 Mar;76(3):272-9., J. McCabe ,et al., article attached as part of the Supporting Documentation]. This is due to the fact that the vaccine is made from the venom of an unrelated species of rattlesnake and the antibodies produced by the vaccinated animal has no protective value as they are unable to bind and neutralize the venom from the bite of a Pacific Rattlesnake. Mr. Brockett and I would like to produce both canine and equine vaccine that would be effective for California and that it would require the use of venom from native rattlesnakes. The logical progression would be to then produce antivenom for domestic animals and ultimately to produce a regionally specific antivenom for humans. The Problem is that under the current regulations it is illegal to commercialize any part of a native animal. This makes it impossible to start a company to produce vaccine, antivenom or any other therapeutic

agents in the state of California using the biochemically unique venoms found in our state's native reptiles. We are proposing that the current regulations be modified or new regulations be written to permit qualified people /companies to collect, process, hold and commercially distribute products produced from the venom of reptiles that are medically relevant and critically needed. This would include provisions for the long term maintenance of venomous reptiles for the purpose of venom extraction and would allow for qualified people/companies to maintain rattlesnakes over the current bag limit. It should be pointed out that the law is currently being violated by the producer of the canine and equine vaccine, Red Rock Biologics, Woodland, CA. The Red Rock vaccine is produced from the venom of a native rattlesnake (Western Diamondback, Crotalus atrox). The only way we could use the existing regulations is to set up a business in another state and simply place an order with a recognized biological supply house for Pacific rattlesnakes. The snakes would be collected and shipped out of state. This would mean that the state of California would be losing business revenue in the form of taxes, employment for California residents and the loss of a company that generates valuable products targeted for use on the west coast. We would prefer to start a business in the state of California and feel it would be an asset to the state. Although we are initially focusing on a vaccine for veterinary use (given prophylactically to companion animals) we also recognize the need for a California specific antivenom for the treatment of snake bites for animals and humans. The following quote from Bryan G. Fry, world renowned herpetologist and venom researcher sums up the current dilemma regarding the treatment of Pacific Rattlesnake bites with the only FDA approved antivenom, CroFab: "In California alone, around 800 people are bitten by rattlesnakes every year. Although just a handful die, the venom is painful, debilitating, and can lead to lengthy hospital stays. To make things worse, Fry says that the antivenom that Americans use for rattlesnake bites—CroFab—is ineffective against the Southern Pacific rattler."It's notoriously poor," he says. "People have to be kept in the hospital for up to a week getting continuous infusions just to keep them alive." There are two problems. First, CroFab uses antibodies that are less allergenic than those in other antivenoms, but get cleared from the body very quickly. "You end up with very expensive urine," says Fry. Second, it doesn't contain antibodies that target the specific proteins used by the Southern Pacific rattlesnake. "They were relying on toxins to be similar to stuff from other rattlesnakes, but even within this one [subspecies], you get completely different venoms. It's been a debacle." [Source: http://phenomena.nationalgeographic.com/2014/01/27/rattlesnakes-two-hours-apart-pack-totally-different-venoms/] We hope that the Fish and Game Commission recognizes the need for and benefit from the commercialization of venom from native reptiles for the production of valuable and potentially lifesaving therapeutic products.

SECTION II: Optional Information

5. Date of Petition: Click here to enter text.

6. Category of Proposed Change

☐ Sport Fishing
☐ Commercial Fishing
☐ Hunting
☒ Other, please specify: The Fish and Game Code, Section: §5061 Rules and Regulations. Authorizes the Fish and Game Commission to establish regulations for commercial take, sale, transport, export or import of native reptiles. CCR, Title 14, Section 651, Commercial Take of

Native Reptiles and Amphibians for Scientific or Educational Institutions. §40. General Provisions Relating to Native Reptiles and Amphibians; (f) Biological Supply Houses and Exempt Organizations.

7. The proposal is to: (*To determine section number(s), see current year regulation booklet or https://govt.westlaw.com/calregs*)

☒ Amend Title 14 Section(s):The Fish and Game Code, Section: §5061 Rules and Regulations. Authorizes the Fish and Game Commission to establish regulations for commercial take, sale, transport, export or import of native reptiles. CCR, Title 14, Section 651, Commercial Take of Native Reptiles and Amphibians for Scientific or Educational Institutions. tific or Educational Institutions. §40. General Provisions Relating to Native Reptiles and Amphibians; (f) Biological Supply Houses and Exempt Organizations

☒ Add New Title 14 Section(s): The Fish and Game Code, Section: §5061 Rules and Regulations. Authorizes the Fish and Game Commission to establish regulations for commercial take, sale, transport, export or import of native reptiles. CCR, Title 14, Section 651, Commercial Take of Native Reptiles and Amphibians for Scientific or Educational Institutions tific or Educational Institutions. §40. General Provisions Relating to Native Reptiles and Amphibians; (f) Biological Supply Houses and Exempt Organizations

☐ Repeal Title 14 Section(s): Click here to enter text.

8. If the proposal is related to a previously submitted petition that was rejected, specify the tracking number of the previously submitted petition Click here to enter text.

Or ☒ Not applicable.

9. Effective date: If applicable, identify the desired effective date of the regulation.

If the proposed change requires immediate implementation, explain the nature of the emergency: Although we would like to begin formulating a new canine/equine vaccine as soon as possible our request would not qualify as an emergency.

10. Supporting documentation: Identify and attach to the petition any information supporting the proposal including data, reports and other documents: We have attached the following documents in support of our petition: **#1.** A publication, co-authored by James McCabe, from the American Journal of Veterinary Research (March 2015, 76(3): 272-279). The significance of this publication is that it shows the current canine rattlesnake vaccine is ineffective against the bite of the most commonly encountered rattlesnake in the state of California and the need for regionally specific vaccines and antivenom produced from the venom of native rattlesnakes. **#2.** A publication from the Journal of Proteomics (March 2014, 99:68-83). The significance of this publication is to draw attention to the extreme variation in venom composition among different populations of Southern Pacific Rattlesnakes. This consequently results in the drastically variable degrees of neutralization by CroFab antivenom. This means that certain patients will receive massive amounts of antivenom with little benefit because it is unable to neutralize the venom due to its different chemical structure. CroFab is the product most commonly used in California (and the United States) to treat snake bites. The variability in venom composition between populations of Pacific Rattlesnakes is also the reason why any successful vaccine or antivenom produced in the future, for California, will require collecting venom from these well-defined populations to insure the product is efficacious. **#3.** Letters of support from two renowned scientists in the field of venom research who recognize the value

in producing regionally specific vaccine and antivenom as well as the valuable resource that would be made available to other researchers if a dependable source of venom was available.

11. Economic or Fiscal Impacts: Identify any known impacts of the proposed regulation change on revenues to the California Department of Fish and Wildlife, individuals, businesses, jobs, other state agencies, local agencies, schools, or housing: The commercialization of venom from native rattlesnakes would have a positive economic impact for the State of California. Venom would be utilized to produce canine and equine vaccines as well as antivenom and other therapeutics for human use. The production of these types of products would create employment opportunities, generate tax revenue for the State and more importantly produce products that would be tailor made to neutralize the effects from the bites of native rattlesnakes. The implementation of new regulations allowing for the commercialization of native rattlesnake venom would most likely have little or no impact on the revenues or expenditures to the California Department of Fish and Wildlife (CDFW). The long term benefits to the State of California would be in the production of vaccines and antivenom that could potentially save lives, reduce pain and suffering of patients and the expense of prolonged hospitalization associated with rattlesnake bites. The commercialization of native rattlesnake venom would also create a legal means by which universities, pharmaceutical and bio-technology companies could secure raw material for future research and development of other beneficial therapeutics.

12. Forms: If applicable, list any forms to be created, amended or repealed:

Not applicable at this time.

SECTION 3: FGC Staff Only

Date received: 11/2/2015

FGC staff action:

☒ Accept - complete
☐ Reject - incomplete
☐ Reject - outside scope of FGC authority

Tracking Number

Date petitioner was notified of receipt of petition and pending action: 11/16/2015

Meeting date for FGC consideration: Dec 2015/ Feb 2016

FGC action:

☐ Denied by FGC
☐ Denied - same as petition _____
Tracking Number
☐ Granted for consideration of regulation change

UNIVERSITY of
NORTHERN COLORADO

College of Natural and Health Sciences
School of Biological Sciences

To: California Department of Fish and Wildlife
From: Dr. Stephen P. Mackessy, Professor of Biology
Re: request for bag limit exemption on California rattlesnakes
Date: 10 April 2015

To whom it may concern:

I am writing this letter in support of James McCabe and Jim Brockett and their plan to develop a new rattlesnake vaccine for dogs and horses that is specifically formulated for California. A recent publication by James McCabe and co-authors has shown that the current vaccine available provides no protection against the bites inflicted by the most commonly encountered species of rattlesnake on the West Coast. The production of such a vaccine will require the maintenance of multiple snakes from many locations within the state, as there are significant geographic variations in venom composition in several species which occur in California. My understanding is that Mr. Brockett and Mr. McCabe are requesting an exemption from the current bag limit regulations for this reason, which I support. This work with California venomous snakes and the vaccine(s) resulting from it will also be a valuable resource for academic and biomedical researchers, as there is currently no supplier of locality-specific rattlesnake venom for species native to California.

Both Mr. Brockett and Mr. McCabe are uniquely qualified to accomplish this goal, as they have a combined total of over 80 years of experience working with venomous reptiles and currently hold permits to maintain non-native venomous snakes. Mr. Brockett is one of the most successful and respected animal trainers in the film industry. Mr. McCabe has worked in biomedical research for over 30 years, and his experience includes working in the Venom Research Laboratory of Dr. Findley Russell at USC.

In conclusion, I believe the proposed project will benefit pets as well as the research community. Please feel free to contact me if you have any questions concerning this letter of support.

Sincerely,

Dr. Stephen P. Mackessy, Professor of Biology
School of Biological Sciences
University of Northern Colorado
501 20th St., CB 92
Greeley, CO 80639-0017 USA

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http://www.unco.edu/nhs/biology/faculty_staff/mackessy/mackessy_stephen.htm

Comparison of the protective effect of a commercially available western diamondback rattlesnake toxoid vaccine for dogs against envenomation of mice with western diamondback rattlesnake (*Crotalus atrox*), northern Pacific rattlesnake (*Crotalus oreganus oreganus*), and southern Pacific rattlesnake (*Crotalus oreganus helleri*) venom

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Received April 11, 2014.
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Address correspondence to Dr. Cates (charles.cates@cshs.org).

OBJECTIVE
To evaluate effectiveness of a commercially available toxoid manufactured from western diamondback (WD) rattlesnake (*Crotalus atrox*) venom against envenomation of mice with WD, northern Pacific (NP) rattlesnake (*Crotalus oreganus oreganus*), and southern Pacific (SP) rattlesnake (*Crotalus oreganus helleri*) venom.

ANIMALS
90 specific pathogen–free female mice.

PROCEDURES
Mice were allocated into 3 cohorts (30 mice/cohort). Mice received SC injections of *C atrox* toxoid (CAT) vaccine (n = 15/group) or adjuvant (15/group) at day 0 and again at 4 weeks. At 8 weeks, mice were challenge-exposed with 1 of 3 venoms. Survival until 48 hours was evaluated by use of log-rank analysis of survival curves and the *z* test for proportions.

RESULTS
6 of 15 WD-challenged CAT-vaccinated mice, 3 of 15 NP-challenged CAT-vaccinated mice, and 0 of 15 SP-challenged CAT-vaccinated mice survived until 48 hours. All adjuvant-only vaccinates survived ≤ 21 hours. Mean survival time of CAT vaccinates was longer than that of adjuvant-only vaccinates for all venoms (1,311 vs 368 minutes for WD, 842 vs 284 minutes for NP, and 697 vs 585 minutes for SP). Results of the *z* test indicated a significantly increased survival rate for vaccinates exposed to WD rattlesnake venom but not for vaccinates exposed to NP or SP rattlesnake venom. Log-rank analysis revealed a significant difference between survival curves of vaccinated versus unvaccinated mice exposed to NP but not WD or SP venom.

CONCLUSIONS AND CLINICAL RELEVANCE
CAT vaccination improved survival rate and survival time after challenge exposure with WD rattlesnake venom and may offer limited protection against NP rattlesnake venom but did not provide significant cross-protection against SP rattlesnake venom. (*Am J Vet Res* 2015;76:272–279)

In 2011, 5,700 incidents of snake envenomation in humans were reported by the American Association of Poison Control Hotlines.[1] The true number of envenomations likely is higher because reporting is not mandatory, many snakebites go unreported, some snake-bite victims do not seek treatment, and some treating physicians do not consult with a poison control center.[2,3] Although the incidence of rattlesnake envenomation in the pet population has not been quantified, it is thought to exceed that for humans (> 150,000 bites/y by 1 estimate[4]) because of a high rate of outdoor exposure, unreported or unnoticed incidents, and a presumed limited-threat judgment for bitten animals.[4,5]

A conditionally licensed WD rattlesnake (*Crotalus atrox*) toxoid vaccine is available for administration to dogs and horses at risk for snakebite and is intended to aid in the reduction of morbidity and deaths attributable to rattlesnake envenomation.[6,7] The authors are not aware of any data on evaluation of the effectiveness of the CAT vaccine in scientific journals.[8] Manufacturer data and advertisements suggest this CAT vaccine is efficacious against bites from WD rattlesnakes and also provides cross-protection against envenomation from other rattlesnake species.[9,a] However, analysis of snake venom reveals it to be a complex milieu of peptides and proteins, and venom from related species and subspecies of rattlesnakes can differ markedly in composition.[10-13] A vaccine that

ABBREVIATIONS

ADE	Antibody-dependent enhancement
CAT	*Crotalus atrox* toxoid
NP	Northern Pacific
OD	Optical density
SP	Southern Pacific
WD	Western diamondback

comprises venom from a single species might provide only limited protection against envenomation by other species of rattlesnakes. In California, companion animals are not typically exposed to WD rattlesnakes because these rattlesnakes are found only in sparsely populated areas in the southeast region of the state. Rather, pets are much more likely to encounter NP rattlesnakes (*Crotalus oreganus oreganus*) and SP rattlesnakes (*Crotalus oreganus helleri*), which inhabit heavily populated and traversed regions of central and coastal California. Therefore, we hypothesized that the CAT vaccine might provide limited cross-protection against 2 important species of rattlesnakes found in California. The purpose of the study reported here was to use rattlesnake envenomation of mice to evaluate the comparative effectiveness of the CAT vaccine against the venom of WD, NP, and SP rattlesnakes.

Materials and Methods

ANIMALS

Ninety specific pathogen–free outbred female Swiss Webster mice (4 to 6 weeks old) were obtained from a commercial source. Mice were allowed to acclimate for 72 hours. Mice were housed in groups (5 mice/cage) on corncob bedding with cotton nesting material in individually ventilated cages in an Association for Assessment and Accreditation of Laboratory Animal Care International–accredited biocontainment facility. All mice were fed standard laboratory rodent chow and provided with ad libitum access to reverse-osmosis-purified acidified water. The room was maintained at 20° to 21°C with relative humidity of 30% to 70%, 10 to 15 air changes/h, and a photoperiod of 12 hours of light to 12 hours of darkness. Use of the mice in this study was approved by the Institutional Animal Care and Use Committee of the University of California-Los Angeles.

EXPERIMENTAL PROCEDURES

A randomized, blinded, placebo-controlled study was conducted. On the basis of an a priori power analysis (power = 0.8, 0% censoring, and 50-to-50 ratio of control mice to experimental mice), the 90 mice were randomly selected by an individual unaffiliated with the study and assigned to treatment and control groups (45 mice/group). Treatment mice received an injection (0.2 mL, SC) of CAT vaccine[b] at day 0 and again at 4 weeks. Control mice received an injection (0.2 mL, SC) of pharmaceutical-grade aluminum hydroxide adjuvant[c] at day 0 and again at 4 weeks. Four weeks after administration of the second injection of CAT vaccine or adjuvant, mice were challenge-exposed with rattlesnake venom.

VENOM

The Society for the Study of Amphibians and Reptiles classification of the western rattlesnake (*Crotalus oreganus*) was used for the present study. The NP and SP rattlesnakes are 2 of 5 recognized subspecies of western rattlesnake, and the WD rattlesnake is a monotypic species with no recognized subspecies. Lyophilized WD rattlesnake venom was obtained.[d] The venom was collected from WD rattlesnakes throughout the range of these rattlesnakes within the United States. Venom of NP and SP rattlesnakes was collected from various regions throughout northern and southern California[14–16] **(Figure 1)**. Samples of NP rattlesnake venom were collected at Sanger (Fresno County), Sutter Butte (Sutter County), Lake Berryessa (Napa County), Vacaville (Solano County), Johnsondale (Tulare County), and Modesto (Stanislaus County). Samples of SP rattlesnake venom were collected at Rasnow Peak, Hidden Valley, Santa Rosa Valley, Carlisle Canyon, Lake Sherwood, and Oak Park (Ventura County); Acton, Castaic, Leona Valley, Topanga Canyon, Malibu Canyon, and Griffith Park (Los Angeles County); Oak Hills, Phelan, Devil's Canyon, and Big Bear (San Bernardino County); Idyllwild-Pine Cove and Garner Valley (Riverside County); and De Luz (San Diego County). Venom samples were processed in accordance with a standardized protocol. The final lyophilized venom product contained equal parts (vol/vol) from each sample location. In preliminary experiments, the LD_{50} was estimated for each venom on the basis of the animal-sparing up-and-down LD_{50} testing paradigm.[17–26] Those LD_{50} values then were used in the study as follows: WD rattlesnake venom, 2.8 mg/kg; NP rattlesnake venom, 1.7 mg/kg; and SP rattlesnake venom, 1.5 mg/kg. These LD_{50} values are similar to those published previously.[27–31]

Figure 1—Map of the distribution for WD rattlesnakes (*Crotalus atrox*; black-shaded area), NP rattlesnakes (*Crotalus oreganus oreganus*; light gray–shaded area), and SP rattlesnakes (*Crotalus oreganus helleri*; dark gray–shaded area) in California and locations for collection of venom samples (circles). The range of each of the rattlesnakes was obtained from previously published information.[14–16] Notice that major metropolitan population centers are located exclusively in the ranges of NP and SP rattlesnakes.

Table 1—Summary of survival data for mice inoculated with CAT vaccine or adjuvant only at 0 and 4 weeks and challenge-exposed 4 weeks later with venom of WD rattlesnakes (*Crotalus atrox*), NP rattlesnakes (*Crotalus oreganus oreganus*), and SP rattlesnakes (*Crotalus oreganus helleri*).

	WD rattlesnake venom		NP rattlesnake venom		SP rattlesnake venom	
Variable	**Vaccine**	**Adjuvant only**	**Vaccine**	**Adjuvant only**	**Vaccine**	**Adjuvant only**
No. of mice injected with venom	15	15	15	15	15	15
No. of mice that survived to 48 h after venom injection	6	0	3	0	0	0
Survival time (min)						
Mean	1,311	368	842	284	697	585
Minimum	121	238	82	160	295	114
Maximum*	2,880	422	2,880	401	1,440	1,269
P value†						
z test for proportions	0.006		0.068		—	
Log-rank analysis	0.146		0.010		0.166	

*An endpoint of 2,880 min (ie, 48 hours) for survival was determined prior to the study (ie, surviving mice were euthanized at 48 hours after venom injection). Despite the fact some mice were expected to live > 48 hours after venom injection, survival time was limited in this manner to avoid effects on reported mean survival times in surviving mice and is in accordance with commonly accepted practices for survival studies.[23] †Values were significant at $P \leq 0.05$.

— = Not applicable because there were no surviving mice in either of these groups.

VENOM CHALLENGE EXPOSURE

Three cohorts (30 mice/cohort [15 treated mice and 15 control mice]) were challenge-exposed with 1 of the 3 venoms at 4 weeks after the second injection of CAT vaccine or adjuvant. Venom was administered to each mouse via IP injection at twice the calculated LD_{50}. For injection, lyophilized venom was reconstituted in sterile water to create a stock solution of 5 mg/mL, which was then diluted as needed to provide the dose for administration. Mice were closely monitored for 48 hours after venom administration.

Before venom administration, body weight and baseline core body temperature were recorded. Temperature was obtained with a 1.5-cm-long thermistor probe inserted via the rectum into the colon; temperature was recorded once per hour for up to 10 hours and thereafter as needed. An observer who was unaware of the venom administered or vaccination status of the mice assessed their condition and determined when a mouse would be euthanized. Mice were euthanized by gradual-fill CO_2 inhalation when they became nonresponsive to stimuli, were in marked respiratory distress (agonal breathing or intermittent gasping), or had a prolonged period of moribundity (severely limited response to stimuli and core body temperature < 70% of the baseline core temperature for > 2 hours). Surviving mice were euthanized 48 hours after venom administration, and a postmortem blood sample was obtained via cardiocentesis.

ANTIBODY TITERS

Blood samples were collected from the retroorbital venous sinus of isoflurane-anesthetized mice 1 week before venom challenge exposure (ie, 3 weeks after the second injection of CAT vaccine or adjuvant) for use in determination of 2 sets of serum antibody titers. First, to verify that mice generated antibodies against the CAT vaccine, serial serum antibody titers of 3 randomly selected vaccinated mice were compared with serial serum antibody titers of 3 randomly selected adjuvant-only control mice. Second, to compare specificity of antibodies generated, dilutions (1:8,000) of serum obtained from 8 randomly selected vaccinated mice were tested against each of the 3 venoms. To generate serial titers and evaluate antibody specificity, 96-well ELISA plates were coated (100 µL/well) with reconstituted venom diluted in 0.1M carbonate buffer (1 µg/mL). Plates were sealed with acetate and incubated overnight at 22°C. After incubation, wells were washed (PBS solution with 0.05% Tween20) and then blocked by incubating on a plate shaker for 15 minutes at 22°C. Diluted serial serum samples were then applied to wells in triplicate. Plates were incubated on a plate shaker for 30 minutes at 22°C. Wells then were washed and horseradish peroxidase–conjugated goat anti-mouse IgG was added; plates were incubated on a plate shaker for 30 minutes at 22°C. Wells were then washed, and the chromogenic substrate tetramethylbenzidine was added. After incubation on a plate shaker for 10 minutes, the reaction was stopped by the addition of 2N sulfuric acid; plates then were immediately evaluated to determine the OD at 450 nm by use of an automated ELISA reader. The OD was used as an indicator of the presence of antivenom IgG as well as for comparisons of relative reactivity between venom types and general assessment of interindividual variation.

STATISTICAL ANALYSIS

Mean survival time in minutes and Kaplan-Meier survival curves were generated for the 3 venoms and saline (0.9% NaCl) solution control samples. A *z* test of proportions was used to compare survival rates of vaccinated versus control mice for all venoms. Log-rank analysis was used to compare Kaplan-Meier survival curves of vaccinated versus control mice for all venoms. Multilevel, mixed-effects linear regression modeling[e] was used to compare specificity of an antibody

titer of 1:8,000 for all venoms. Significance for all tests was set at $P \leq 0.05$.

Results

SURVIVAL RATE AND SURVIVAL TIME

Both survival rate and survival time were analyzed **(Table 1)**. For mice vaccinated with CAT vaccine, 6 of 15 mice challenge-exposed with WD rattlesnake venom, 3 of 15 mice challenge-exposed with NP rattlesnake venom, and 0 of 15 mice challenge-exposed with SP rattlesnake venom were alive at 48 hours after venom injection, whereas adjuvant-only control mice survived ≤ 21 hours after injection of any of the 3 rattlesnake venoms. Mean survival time of vaccinated mice was longer than that of adjuvant-only control mice for all venoms (1,311 vs 368 minutes for WD rattlesnake venom, 842 vs 284 minutes for NP rattlesnake venom, and 697 vs 585 minutes for SP rattlesnake venom). Survival analysis for individual venom revealed that results of the z test for proportions were significant ($P = 0.01$) only for WD rattlesnake venom. Log-rank analysis of survival curves revealed significant ($P = 0.01$) differences only for NP rattlesnake venom **(Figure 2)**. Maximum survival time was greatest for vaccinated mice, compared with survival time for adjuvant-only control mice, for all venoms. Notably, minimum survival time was greater for control mice than for vaccinated mice for both WD and NP rattlesnake venoms. This was evident on the Kaplan-Meier survival curve for WD rattlesnake venom as an initial increase in death of vaccinated mice, compared with that of control mice, at early time points (< 300 minutes after venom injection). Because of this finding, a log-rank analysis for WD rattlesnake venom that excluded early time points was conducted (n = 7 mice) and revealed a significant ($P = 0.004$) effect.

Student t test analysis of prestudy mean body weight and baseline core body temperature revealed that these variables did not differ significantly among any of the groups ($P = 0.08$ to 0.67; data not shown). No morbidity or deaths were associated with receiving the vaccine or adjuvant alone.

ANTIBODY TITERS

Antibody titers against all 3 rattlesnake venoms for the 3 vaccinated and 3 control mice were plotted **(Figure 3)**. Dilutions tested were 1:4,000, 1:8,000, 1:16,000, 1:32,000, 1:64,000, and 1:128,000. Mice vaccinated with CAT developed measurable antibody titers against all 3 venoms, whereas mice receiving only adjuvant had no evidence of reactive serum antibodies against any venom. The OD for a 1:8,000 dilution of serum obtained from 8 additional randomly selected vaccinated mice tested against all 3 venoms was plotted **(Figure 4)**. Comparison of OD for the various venoms suggested a decreasing reactivity as follows: the reactivity of WD rattlesnake venom was greater than that of NP rattlesnake venom, and the reactivity of NP rattlesnake venom was greater than that of SP rattlesnake venom. Analysis of a multilevel mixed-effects linear regression model with venom as the sole categorical predictor revealed significant ($P \leq 0.001$) differences in OD for each venom. Interindividual variation was also evident because the majority (6/8) of the mice had titers with OD values approaching or exceeding 1.0, whereas the remainder (2/8) had OD values < 0.5.

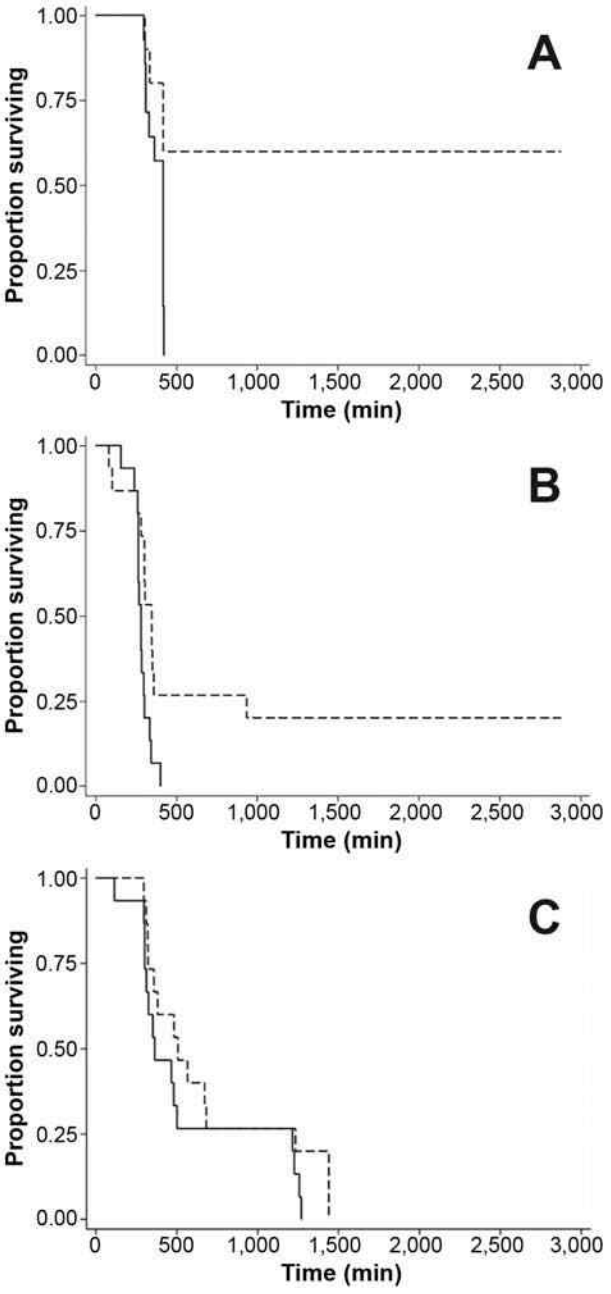

Figure 2—Kaplan-Meier survival curves for vaccinated mice (dashed lines) and adjuvant-only control mice (solid lines) after challenge exposure with WD rattlesnake venom (A), NP rattlesnake venom (B), and SP rattlesnake venom (C). There were 15 mice in each group. Time of challenge exposure (injection of venom) was designated as time 0. There was a significant ($P = 0.01$; log-rank analysis) difference in survival curves of vaccinated versus adjuvant-only mice after injection of only NP rattlesnake venom. In panel A, notice the possible early death phenomenon attributable to ADE of WD rattlesnake venom.

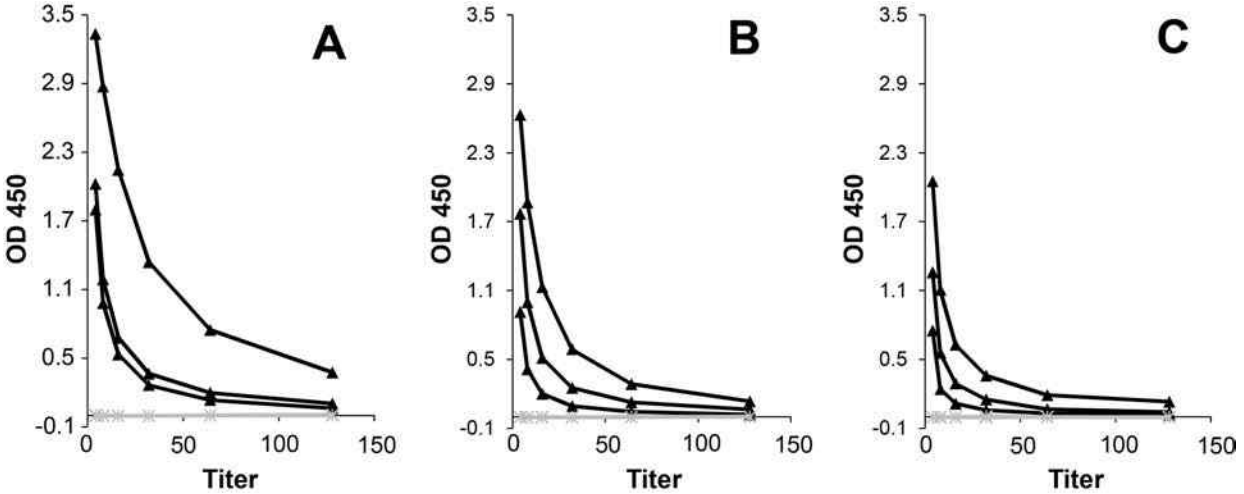

Figure 3—Serial serum dilution antibody titers for 3 vaccinated mice (black symbols) and 3 adjuvant-only control mice (gray symbols) against venom of WD rattlesnakes (A), NP rattlesnakes (B), and SP rattlesnakes (C) as determined by OD measured at 450 nm (OD 450). Each black symbol represents results for 1 mouse; the gray symbol represents results for 3 mice. Notice that the antibody response of vaccinated mice was greater than that of the control mice for all venoms. There was a pattern that specificity (ie, increased OD 450) was greater against venom of WD rattlesnakes than against venom of NP or SP rattlesnakes. The x-axis represents a dilution factor of 1:1,000. Dilutions tested were 1:4,000, 1:8,000, 1:16,000, 1:32,000, 1:64,000, and 1:128,000.

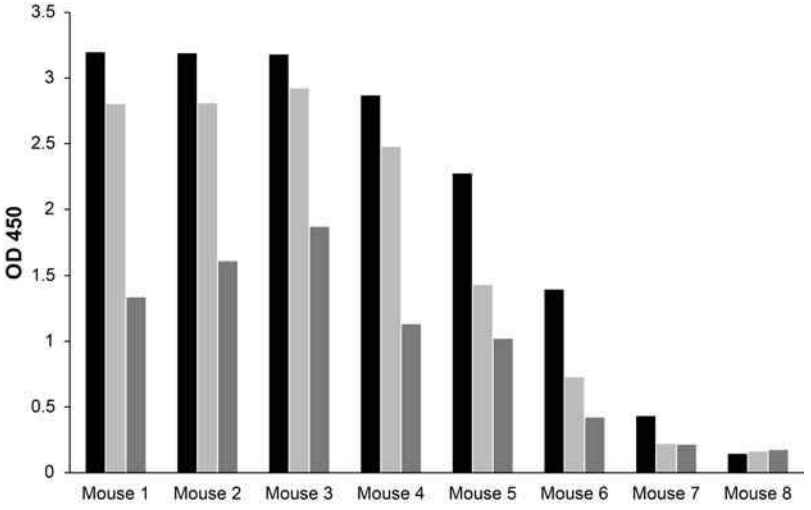

Figure 4—Single serum dilution (1:8,000) antibody titers for 8 randomly selected mice against venom of WD rattlesnakes (black bars), NP rattlesnakes (light gray bars), and SP rattlesnakes (dark gray bars). Notice the marked interindividual differences as well as differences in specificity among venoms (WD rattlesnake > NP rattlesnake > SP rattlesnakes venom). There was a significant ($P \leq 0.001$; multilevel mixed-effects linear regression) difference in OD 450 among venoms.

Discussion

In the present study, survival analysis after rattlesnake envenomation of mice was conducted in a randomized, blinded, placebo-controlled study to evaluate the comparative effectiveness of CAT vaccine against 3 rattlesnake venoms. The data reported included evaluation of survival rate (whether a mouse died ≤ 48 hours after venom injection) as well as evaluation of survival time (number of minutes a mouse survived after venom injection, up to 48 hours). Survival time is an important consideration in light of the fact a venom vaccine may be useful if it extends the course of the envenomation, thereby allowing additional time to seek primary medical treatments such as antivenin and intensive care. In addition, antibody titers of vaccinated and adjuvant-only control mice were compared as well as specificity of the antibodies generated against each of the 3 venoms. Overall, results of the challenge-exposure experiment indicated that CAT vaccination resulted in a significant increase in survival rate and survival time against injection with WD rattlesnake venom; equivocal results after injection of NP rattlesnake venom, which would likely require a greater number of mice to verify a difference; and no significant improvement in survival measures after injection of SP rattlesnake venom. Analysis of antibody titers revealed a clearly measurable antibody response in vaccinated mice, compared with that in adjuvant-only control mice, against all 3 venoms. The antibodies were most reactive against WD rattlesnake venom, with significantly less reactivity against venoms of the 2 other rattlesnake species.

Analysis of the data for the present study indicated that administration of CAT vaccine conferred an increase in survival rate and survival time in vaccinated versus control mice challenge-exposed with WD rattlesnake venom. Mean survival time was greater in vaccinated than in control mice, and survival rate improved significantly ($P = 0.01$; z test for proportions). Unexpectedly, results for log-rank analysis of

survival curves did not reveal significant differences. This result was particularly surprising because challenge exposure with NP rattlesnake venom had a significant effect, as determined by use of log-rank analysis, despite the fact there were only half as many survivors as for challenge exposure with WD rattlesnake venom. Notably, minimum survival time was greater for control versus vaccinated mice for both WD and NP rattlesnake venom (Table 1). This was also evident on the Kaplan-Meier survival curve for WD rattlesnake venom as an initial increase in death of vaccinated versus adjuvant-only control mice at early time points (< 300 minutes after venom injection; Figure 2). The early deaths may have sufficiently altered early time points of the curve of vaccinated mice after injection of WD rattlesnake venom such that statistical modeling resulted in a curve for vaccinated mice that was indiscernible from the curve for the control mice, despite the clear difference at later time points ($P = 0.004$ for log-rank analysis after 300 minutes). We propose that the early deaths could have been attributable to 1 factor or a combination of factors, such as genetic predisposition to venom sensitivity, injection near or into a vascular bed that hastened systemic exposure to venom, or an antibody-mediated early death phenomenon that has been observed in a laboratory setting when testing vaccines against viruses and bacterial toxins.[32–39]

Use of the vaccine may afford limited cross-protection against NP rattlesnake venom; however, the data are not entirely conclusive. Mean survival rate of vaccinated mice significantly ($P = 0.01$; log-rank analysis of survival curves) exceeded that of adjuvant-only control mice, which suggested a protective effect. However, results of the z test for proportions of survival time did not reveal significant ($P = 0.07$) differences. However, it is plausible that testing a larger population of mice may have allowed us to detect a more subtle effect by use of the z test of proportions.

The vaccine did not provide significant protection against SP rattlesnake venom, although the mice with the greatest survival time were in the vaccinated group. The CAT vaccine may have been less effective against SP rattlesnake venom because of the divergent molecular composition of that venom. For example, 1 population of SP rattlesnakes can produce Mojave toxin, a unique and powerful neurotoxin, which to date has not been found in WD or NP rattlesnake venoms.[15,40]

In addition to survival analysis, antibody titers were measured in a number of mice to verify an antibody response against the CAT vaccine (Figure 3). Compared with control mice, vaccinated mice had a variably robust antibody response, and initial titers suggested that the antibodies were more specific for WD rattlesnake venom than for the NP or SP rattlesnake venoms. On the basis of this observation, sera from 8 randomly selected vaccinated mice were evaluated for antibody specificity against each of the 3 venoms evaluated in the study (Figure 4). Linear regression analysis revealed significantly increased OD against WD rattlesnake venom, as compared with results against SP or NP rattlesnake venoms. The analysis indicated that antibodies generated by mice were most specific against the venom of manufacture (ie, WD rattlesnake venom), compared with specificity against the other 2 genetically distinct venoms. It should be emphasized that antibody titers were measured only to verify that mice generated an antibody response against the vaccine and to evaluate the specificity of that antibody response. The magnitude of the murine antibody response and how it may relate to survival of vaccinated dogs and horses (or the ability of clinicians to provide a prognosis for survival of vaccinated animals) in real-life situations were beyond the scope of the present study.

The present study had several potential confounders. First, on the basis of a previous manufacturer-designed study,[a] mice in the present study were injected with a vaccine dose of 0.2 mL, which could be from 50- to 1,500-fold as high (by volume) as manufacturer-recommended doses for dogs and horses.[6,7] Potentially, this could have resulted in a more robust antibody response and more enhanced protective benefit than typically would be afforded to companion animals. On the other hand, it should be mentioned that mice were challenge-exposed with an extremely high (twice the LD_{50}) dose of venom administered via the IP route commonly used in venom studies on mice. In most naturally occurring scenarios, companion animals receive SC or IM injection of venom, which results in slower and less immediately severe systemic effects[41] than were seen in the mice of the study reported here. In light of this, findings for the present study should be considered with the caveat that, in theory, the vaccine may improve survival rate and survival time, but these improvements remain to be definitively verified in practice settings for the specific species and situations of interest. Finally, it should be mentioned that we evaluated survival rate and survival time but did not directly assess morbidity. In actual envenomations, local effects such as severe necrosis, hemorrhage, and inflammation can cause substantial morbidity, which potentially can lead to severe incapacitation and death.[42–45] It remains to be determined whether vaccination has substantial effects to prevent or reduce important local sequelae after snake envenomation. Despite these drawbacks, there are a number of reasons investigators should use the described method of envenomation of mice, including that it is a well-accepted technique for venom analysis and antivenin evaluation, adheres to the concept of replacement in research (ie, use of mice instead of dogs or horses), and has been used in experiments conducted by the manufacturer to obtain USDA licensing for the CAT vaccine.

Data from the rattlesnake envenomation of mice reported here indicated that administration of the CAT vaccine resulted in a significant increase in survival

rate and survival time after injection of WD rattlesnake venom, equivocal results after injection of NP rattlesnake venom (possibly requiring a greater number of animals to confirm a difference), and no significant improvement in survival variables after injection of SP rattlesnake venom. Analysis of antibody titers confirmed a measurable antibody response in vaccinated versus adjuvant-only control mice and confirmed that specificity of the antibody response was significantly greater against the venom of manufacture. Overall, results of the present study suggested that vaccination with the CAT vaccine may provide limited cross-protection against NP rattlesnake venom but no significant cross-protection against SP rattlesnake venom. Future studies should include more in-depth analysis of antibody titers, testing of alternative vaccination strategies involving other venoms, and investigation into early deaths seen in some of the vaccinated mice. Such studies will be useful in validating results of the present study and providing increased insight into the real-world effectiveness of a rattlesnake venom vaccine.

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Footnotes

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d. Lot No. CAT 1412, Kentucky Reptile Zoo, Slade, Ky.
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5 June 2015

To whom it concerns,

I am writing this letter in support of James McCabe and Jim Brockett, who intend to develop a new rattlesnake vaccine for dogs and horses that is specifically formulated for California snake species. The current vaccine, produced by Red Rock Biologics, consists of heat-treated Western Diamondback Rattlesnake (*Crotalus atrox*) venom. My own unpublished research shows that this venom is highly dissimilar to that of most California rattlesnake species (the Red Diamond Rattlesnake, *Crotalus ruber*, being an exception), and therefore will not generate protective antibodies to other rattlesnake venoms. Indeed, a recent publication by James McCabe has shown that the current vaccine provides no protection against the bites inflicted by the most commonly encountered species of rattlesnake on the west coast, *Crotalus oreganus*.

The production of such a vaccine will require the maintenance of multiple snakes from many locations within the state, as there are geographic variations in venom composition. My understanding is that Mr. Brockett and Mr. McCabe are requesting an exemption from the current bag limit regulations for this reason. In addition to their need for production of this vaccine, their extraction of venom from these snakes would also comprise a valuable resource for other academic and biomedical researchers, as there is currently no existing supplier of locality-specific rattlesnake venom for species native to California. I will share with Mr. Brockett and Mr. McCabe my unpublished data on geographic variation in venom of all California rattlesnake taxa so that they can plan accordingly which geographic locations to focus on, thereby reducing the total number of snakes they would need to sample and maintain (though the number will still exceed the current limit).

Both Mr. Brockett and Mr. McCabe are uniquely qualified to accomplish this goal, as they have a combined total of over 80 years of experience working with venomous reptiles and currently hold permits to maintain nonnative venomous snakes. Mr. Brockett is one of the most successful and respected animal trainers in the film industry. Mr. McCabe has worked in biomedical research for over 30 years. His experience includes working in the Venom Research Laboratory of Dr. Findley Russell at USC, and he has graciously provided my own laboratory with venom samples.

In conclusion, I believe the proposed project will benefit pets as well as the research community. Please feel free to contact me if you have any questions regarding this letter of support.

All best wishes,

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Intraspecific venom variation in the medically significant Southern Pacific Rattlesnake (*Crotalus oreganus helleri*): Biodiscovery, clinical and evolutionary implications

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ABSTRACT

Due to the extreme variation of venom, which consequently results in drastically variable degrees of neutralization by CroFab antivenom, the management and treatment of envenoming by *Crotalus oreganus helleri* (the Southern Pacific Rattlesnake), one of the most medically significant snake species in all of North America, has been a clinician's nightmare. This snake has also been the subject of sensational news stories regarding supposed rapid (within the last few decades) evolution of its venom. This research demonstrates for the first time that variable evolutionary selection pressures sculpt the intraspecific molecular diversity of venom components in *C. o. helleri*. We show that myotoxic β-defensin peptides (aka: crotamines/small basic myotoxic peptides) are secreted in large amounts by all populations. However, the mature toxin-encoding nucleotide regions evolve under the constraints of negative selection, likely as a result of their non-specific mode of action which doesn't enforce them to follow the regime of the classic predator–prey chemical arms race. The hemorrhagic and tissue destroying snake venom metalloproteinases (SVMPs) were secreted in larger amounts by the Catalina Island and Phelan rattlesnake populations, in moderate amounts in the Loma Linda population and in only trace levels by the Idyllwild population. Only the Idyllwild population in the San Jacinto Mountains contained potent presynaptic neurotoxic phospholipase A_2 complex characteristic of Mohave Rattlesnake (*Crotalus scutulatus*) and Neotropical Rattlesnake (*Crotalus durissus terrificus*). The derived heterodimeric lectin toxins characteristic of viper venoms, which exhibit a diversity of biological activities, including anticoagulation, agonism/antagonism of platelet activation, or procoagulation, appear to have evolved under extremely variable selection pressures. While most lectin α- and β-chains evolved rapidly under the influence of positive Darwinian selection, the β-chain lectin of the

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Catalina Island population appears to have evolved under the constraint of negative selection. Both lectin chains were conspicuously absent in both the proteomics and transcriptomics of the Idyllwild population. Thus, we not only highlight the tremendous biochemical diversity in *C. o. helleri*'s venom-arsenal, but we also show that they experience remarkably variable strengths of evolutionary selection pressures, within each toxin class among populations and among toxin classes within each population. The mapping of geographical venom variation not only provides additional information regarding venom evolution, but also has direct medical implications by allowing prediction of the clinical effects of rattlesnake bites from different regions. Such information, however, also points to these highly variable venoms as being a rich source of novel toxins which may ultimately prove to be useful in drug design and development.

Biological significance

- These results have direct implications for the treatment of envenomed patients.
- The variable venom profile of *Crotalus oreganus helleri* underscores the biodiscovery potential of novel snake venoms.

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1. Introduction

Knowledge of venom composition has increased dramatically with improvements in technology and the advent of new techniques, in particular the use of mass spectrometry in venom proteomics [1–15] and venom gland transcriptome analysis [16–27]. Snake venoms are complex secretions composed of numerous enzymes, toxins, peptides, small organic molecules, and inorganic components that have diverse modes of action on both prey and human victims [28–32]. Snake venom serves both predatory and defensive purposes [28–30,33–38]. Variation in venom profiles has been shown between species within the same genus [5,11,12,15,27,39–44] and between individuals within the same species, with the intraspecific differences found among geographic locales [2,11,12,45–52], between sexes [46,47,53] and between juveniles and adults [9,46,47,54,55]. Venom variation has also been reported between venom glands of a single individual [56]. Some authors have argued that venom diversity is the product of neutral evolutionary processes and not subject to natural selection [57,58], whereas others have argued that strong natural selection has driven adaptation to particular prey species [12,30,31,40,46,47,59–63].

Venom in reptiles originated from a single early recruitment event approximately 180 million years ago (mya) during the early Jurassic period and is a plesiotypic trait of the Toxicofera clade [10,12,18,20–23,30,31,40,64]. New World pit vipers are thought to have descended from a single ancestral Asian pit viper species that colonized the New World via the Bering land bridge [65,66], with rattlesnakes having a mid-Cenozoic origin in the Mexican highlands [67–69]. The venom arsenals of Crotaline snakes are characterized by a great diversity of venom-components; generalized venom "types" have been proposed, depending upon metalloprotease activity and toxicity [70]. Type I venoms possess high levels of metalloprotease activity and lower toxicity (>1.0 μg/g mouse body weight), whereas type II venoms have low metalloprotease activity and higher toxicity (<1.0 μg/g mouse body weight). The presence of these two venom types in a diversity of well-defined species clades suggests that it is not dependent upon phylogeny [49,52,70–72].

Crotalus oreganus helleri is a medium-sized rattlesnake inhabiting Baja California northward through southern California, and the Pacific islands of Santa Catalina (Los Angeles County, California) and Coronado Del Sur (Tijuana, Mexico) [67]. Pronounced tectonic activity in the region has produced considerable variation in available habitat [73]. The species utilizes habitat ranging from sea level to >3000 m and prey encountered are highly varied. Significant regional variation in venom composition exists [51,74], with both type I and type II venoms identified in local populations [49]; however this dichotomy of venom types fails to characterize the full extent of venom variability in the species. *C. o. helleri* is the most medically relevant species of the region and is responsible for the majority of severe envenomations in southern California [29,75]. Therefore, determining intraspecific variation of *C. o. helleri* venom components and the factors influencing their molecular evolution can yield important implications for clinical treatment of envenomation. Venom variation also offers substantial potential for bioprospecting and pharmaceutical discovery [8,18–23,30,40,76]. These variations have been the subject of many popular press reports that grossly misattribute them to unparalleled recent diversification of the venom [77] and thus display a fundamental lack of understanding on how venom evolves.

In this study, we investigated the diversity of toxins present in *C. o. helleri*, across its geographic range, using a combined proteomics–transcriptomics approach to investigate the relative molecular evolution and diversification within a given toxin type, and the relative expression levels of particular toxin types.

2. Materials and methods

2.1. Sampling

We sampled four southern California populations of *C. o. helleri* from areas with pronounced geological, elevational, and floristic differences. Human envenomations from snakes in these

different regions have exhibited distinct symptoms ranging from hemorrhage to muscle fasciculations to paralysis. The four populations chosen (Fig. 1) were: (1) Catalina Island, which is dominated by coastal sage scrub, interspersed with chaparral and oak woodland, has never been connected to the mainland [73] and has supported an isolated population since at least the Pleistocene; (2) Idyllwild in the San Jacinto Mountains has high altitude pine and cedar montane forests (elevation ~1600 m); (3) Loma Linda consists of low rolling hills covered with grasses and, on north facing slopes, *Salvia mellifera* and other shrubs; and (4) Phelan comprises a transition zone between High Desert (Mohave) and coastal mountain scrub. We sampled one snake from each region for transcriptome sequencing. We used the same snake for proteome analysis of the Phelan and Loma Linda populations, and a separate individual of same sex and size from the exact same locality for the other two locations in addition to two more specimens for each location other than Loma Linda, for which only one more specimen was obtained due to the rarity of *C. o. helleri* in this location. We used only adult specimens for venom analysis due to potential ontogenetic shifts in venom composition [9,70].

2.2. Transcriptome sequencing, phylogenetics, selection analyses, and structural analyses

2.2.1. Transcriptome sequencing

Total RNA was extracted from venom glands using the standard TRIzol Plus method (Invitrogen). Extracts were enriched for mRNA using standard RNeasy mRNA mini kit (Qiagen) protocol. mRNA was reverse transcribed, fragmented and ligated to a unique 10-base multiplex identifier (MID) tag prepared using standard protocols and applied to one PicoTitrePlate (PTP) for simultaneous amplification and sequencing on a Roche 454 GS FLX + Titanium platform (Australian Genome Research Facility). An average of 50,000 sequences were read for each library. Automated grouping and analysis of sample-specific MID reads informatically separated sequences from the other transcriptomes on the plates, which were then post-processed to remove low quality sequences before de novo assembly into contiguous sequences (contigs) using v 3.4.0.1 of the MIRA software program. Assembly details for the transcriptomes are shown in Supplementary Table 1. All raw reads have been deposited in the NCBI Sequence Read Archive (http://www.ncbi.nlm.nih.gov/sra/) with the accession numbers of: SRR871501 *C. o. helleri* (Catalina Island), SRR871502 *C. o. helleri* (Idyllwild), SRR871503 *C. o. helleri* (Loma Linda), and SRR871504 *C. o. helleri* (Phelan). Assembled contigs were processed using CLC Main Work Bench (CLC-Bio) and Blast2GO bioinformatic suite to provide Gene Ontology, BLAST and domain/Interpro annotation. The above analyses assisted in the rationalization of the large numbers of assembled contigs into phylogenetic 'groups' for detailed phylogenetic analyses outlined below.

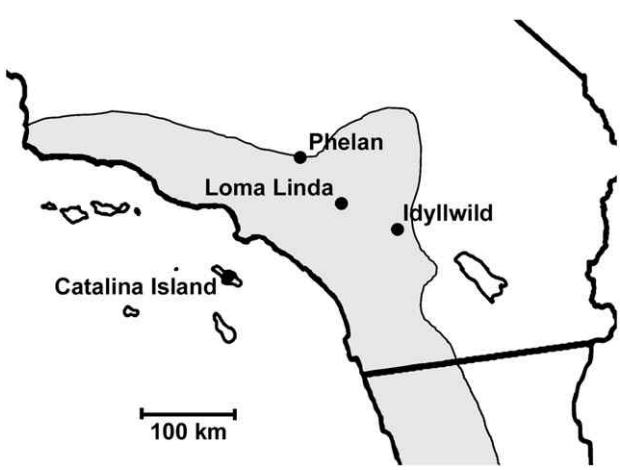

Fig. 1 – *C. o. helleri* populations investigated.

2.2.2. Selection analyses

Translated nucleotide sequences were aligned using MUSCLE 3.8 [78] and the alignments were manually inspected to rectify errors. All nucleotide sequences and multiple sequence alignments used for selection analyses are available as Supplementary file 2 and Supplementary Figs. 1–4, respectively. In order to reconstruct gene phylogenies for selection assessments, maximum-likelihood method implemented in PhyML [79] was employed on the nucleotide datasets and node support was evaluated with 1000 bootstrapping replicates. All the maximum-likelihood trees are provided as Supplementary Figs. 5–7, with the results of branch-site REL test mapped onto them. In order to detect the nature of selection and its influence on various venom-encoding genes of *C. o. helleri*, we utilized maximum-likelihood models implemented in Codeml of the PAML [80]. We employed site-specific models that estimate positive selection statistically as a non-synonymous-to-synonymous nucleotide-substitution rate ratio (ω) significantly greater than 1. For technical details regarding models/methods see [20,81]. FUBAR [82] implemented in HyPhy [83] was employed to provide additional support to the aforementioned analyses and to detect sites evolving under the influence of pervasive diversifying and purifying selection pressures. Mixed Effects Model Evolution (MEME) [82] was also employed to efficiently detect episodically diversifying sites. To clearly depict the proportion of sites under different regimes of selection, an evolutionary fingerprint analysis was carried out using the evolutionary selection distance (ESD) algorithm implemented in Datamonkey [84]. We further utilized the branch-site Random Effects Likelihood (REL) test [85] to identify lineages evolving under the influence of episodic diversifying selection pressures.

2.2.3. Structural analyses

To depict the natural selection pressures influencing the evolution of various *C. o. helleri* venom-components (only those with sufficient numbers of full-length sequences were analyzed in this regard: β-defensin, kallikrein and lectin), we mapped the sites under positive selection on the homology models created using Phyre 2 web server [86]. PyMOL 1.3 [87] was used to visualize and generate the images of homology models. ConSurf web server [88] was used for mapping the evolutionary selection pressures on the three-dimensional homology models.

Homology models of the presynaptic PLA_2 complex from *C. o. helleri* (Coh) (GenBank: GAKR01000015 [acid subunit] and GenBank: GAKR01000016 [basic subunit]) and the homologue from *Crotalus scutulatus scutulatus* (Css) (UniProt: P18998 [acid subunit] and UniProt: P62023 [basic subunit]) were built using the crystal structure of crotoxin from *Crotalus durissus terrificus* (Cdt)

(PDB: 3R0L; UniProt: P08878 [acid subunit]; UniProt: P0CG56 [basic subunit]) [89] as a template. Template to sequence alignments were generated using SPDBV [90,91] and exported as FASTA-formatted text. The 3R0L coordinates together with the alignment file were used for comparative modeling using MODELLER [92]. Images of these homology models were obtained using VMD [93] and Tachyon ray tracing. Charged surfaces were obtained by running the Adaptive Poisson–Boltzmann Solver APBS plug-in [94] to VMD. Representation in VMD was set to "orthographic", depth cueing was set to "off", and render mode was set to "GLSL".

2.3. Proteomics

2.3.1. HPLC

Lyophilized crude venom was diluted to a concentration of 3 mg/mL in Buffer A (0.065% TFA, 2% acetonitrile in Nanopure water) and centrifuged at 15,000 *g* for 10 min. The supernatant (100 μL) was fractionated on an ÄKTAmicro high-pressure liquid chromatography (HPLC) system (GE Healthcare Life Sciences, Piscataway, NJ, USA) fitted with two reversed-phase (RP) columns (SOURCE 5RPC ST polystyrene/divinyl benzene, 4.6 × 150 mm; GE Healthcare) run in series at a flow rate of 0.5 mL/min, using a linear gradient of 0–100% Buffer B (0.05% TFA, 80% acetonitrile in Nanopure water) over 40 column volumes. Protein elution was monitored at 214 nm using Unicorn 5.0 (GE Healthcare Life Sciences) software, and fractions were collected manually.

2.3.2. LC–MS

Each fraction was subjected to reduction and alkylation prior to enzymatic digestion using dithiothreitol and iodoacetamide, respectively, following the protocol outlined by Matsudaira [95]. Proteins were then digested with proteomics-grade porcine pancreatic trypsin (Sigma-Aldrich, St. Louis, MO, USA). We desalted samples using C_{18} ZipTips (EMD Millipore, Billerica, MA, USA) according to the manufacturer's protocol. The desalted tryptic peptides were resuspended in mobile phase A (2% acetonitrile, 0.1% formic acid in water). Liquid chromatography was conducted on a ThermoFinnigan LCQ Deca XP spectrometer (ThermoFinnigan, Waltham, MA, USA) equipped with a PicoView 500 nanospray apparatus using Xcalibur software (ver. 1.3; ThermoFinnigan, Waltham, MA, USA) for instrument control and data acquisition. Separation was performed on a 10-cm × 75-μm-i.d. C18 BioBasic bead column (New Objective, Woburn, MA, USA) by injecting 20-μL samples. Mobile phase B consisted of 98% acetonitrile, 2% water, and 0.1% formic acid. The gradient program was: 0% B at 0.18 mL/min for 7.5 min; 0% B at 0.35 mL/min for 0.5 min; linear gradient to 20% B at 15 min at 0.35 mL/min; linear gradient to 75% B at 55 min at 0.3 mL/min (flow rate constant for remainder of the program); linear gradient to 90% B at 60 min; hold at 90% B until 85 min; linear gradient to 0% B at 90 min; hold at 0% B until 120 min. Spectra were acquired in positive ion mode with a scan range of 300–1500 m/z. We converted MS/MS data into peak list files using ExtractMSn implemented in BioWorks (version 3.1; ThermoFinnigan) with the following parameters: peptide molecular weight range of 300–3500, threshold of 100,000, precursor mass tolerance of 1.4, and minimum ion count of 35. We conducted MS/MS database searches using Mascot (licensed, version 2.2, Matrix Science, Boston, MA, USA) against the National Center for Biotechnology Information non-redundant (NCBInr) database in the taxon Metazoa with a parent tolerance of 1.20 Da, fragment tolerance of 0.60 Da, and two missed trypsin cleavages allowed. We

Table 1 – *C. o. helleri* intraspecific proteomic and transcriptomic toxin presence.

Toxin molecular scaffold type	Catalina Island		Idyllwild		Loma Linda		Phelan	
	P	T	P	T	P	T	P	T
β-defensin	Large amounts, medium complexity	✓	Large amounts, medium complexity	✓	Large amounts, medium complexity	✓	Large amounts, medium complexity	✓
CNP-BPP	Medium amounts, low complexity	✓	Low amounts, low complexity	✓	Large amounts, low complexity	✓	Large amounts, low complexity	✓
CRiSP	Large amounts, medium complexity	✓	✖	✓	Medium amounts, low complexity	✓	Medium amounts, medium complexity	✖
Hyaluronidase	✖	✓	✖	✓	✖	✓	✖	✓
Kallikrein	Medium amounts, low complexity	✓	Medium amounts, high complexity	✓	Large amounts, high complexity		Large amounts, high complexity	✓
Kunitz	✖	✓	✖	✓	✖	✓	✖	✓
L-Amino acid oxidase	Medium amounts, low complexity	✓	Medium amounts, low complexity	✓	Medium amounts, low complexity	✓	Medium amounts, low complexity	✓
Lectin	Large amounts, medium complexity	✓	✖	✖	✖	✓	Low amounts, low complexity	✓
Nerve growth factor	Low amounts, low complexity	✓	✖	✓	Low amounts, low complexity	✓	✖	✖
Phospholipase A_2	Medium amounts, medium complexity	✓	Large amounts, high complexity	✓	Low amounts, low complexity	✓	Low amounts, low complexity	✓
Snake venom metalloprotease	Large amounts, medium complexity	✓	Not detected	✓	Medium amounts, high complexity	✓	Large amounts, high complexity	✓
Vascular endothelial growth factor	✖	✓	✖	✓	Low amounts, low complexity	✓	Low amounts, low complexity	✓
Vespryn	✖	✓	✖	✓	✖	✓	✖	✓

P = proteome.
T = transcriptome.

specified carbamidomethylation of cysteine and oxidation of methionine in Mascot as fixed and variable modifications, respectively.

2.3.3. MALDI ToF MS and MALDI ToF/ToF MS/MS

RP-HPLC fractions were submitted to the Institute for Integrated Research in Materials, Environments and Society at California State University, Long Beach, to determine whole protein molecular masses and protein identification/similarity. For MALDI ToF/ToF MS/MS analysis, tryptic peptides were mixed with α-cyano-4-hydroxy cinnamic acid (CHCA) matrix and directly spotted onto MALDI plates. MS spectra were collected using 1000 laser shots/spectrum, and MS/MS spectra from 3000 shots/spectrum. Peptides with signal-to-noise ratio above 15 in MS mode were selected for MS/MS analysis, with a maximum of 15 MS/MS spectra allowed per spot. Internal calibration was achieved using ToF/ToF Calibration Mixture (AB SCIEX). We searched MS/MS data against the NCBInr database within Metazoa using GPS Explorer, running Mascot (version 2.1) search engine with a peptide tolerance of 300 ppm, MS/MS tolerance of 0.8 Da, and one missed cleavage allowed. We specified carbamidomethylation of cysteine as a fixed modification, and the following as variable modifications: carbamyl, Gln/pyro-Glu (N-term Q), and Glu/pyro-Glu (N-term E). Mass spectrometry data for the peaks in Supplementary File 1 is presented in Supplementary Spreadsheet 1.

2.3.4. Statistical analyses

To confirm that population differences existed among the 11 snakes with the quantitative RP-HPLC data presented in Supplementary Spreadsheet 2, we subjected the percent protein present in each of the 11 toxin families (area under the peaks) to a 4 × 11 (population × toxin family) analysis of variance (ANOVA [96]), treating population as a between-subjects factor and toxin family as a within-subjects factor. We rank-transformed the data to avoid analysis of percentage data that summed to 100 for

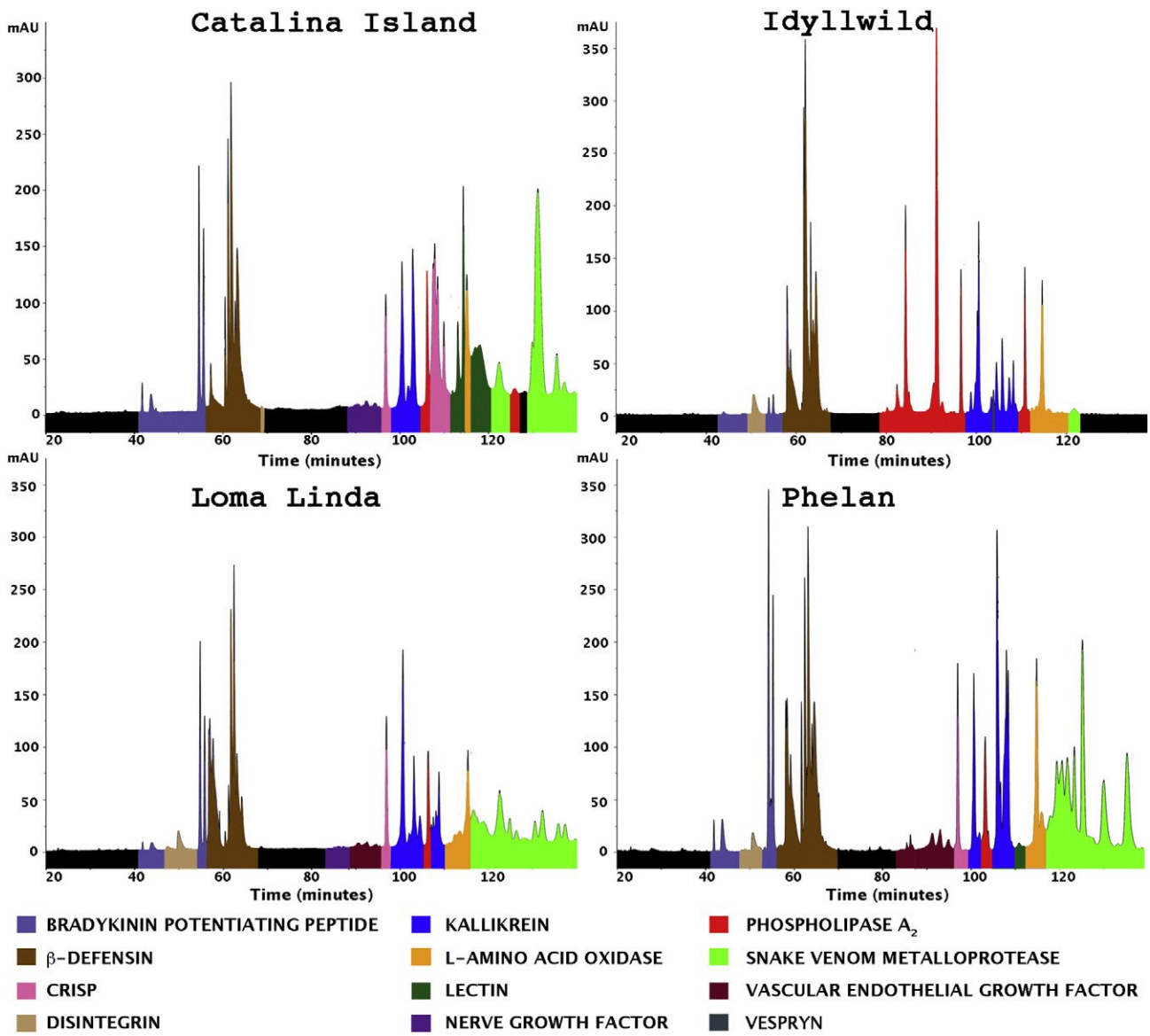

Fig. 2 – LC–MS/MS annotated RP-HPLC chromatograms from the four different *C. o. helleri* populations examined in this study.

each individual. Although our samples were small and data were somewhat non-normal and heteroscedastic, general linear models generally handle data well that fail to meet parametric assumptions and the results were extremely robust. We also ran a non-parametric Kruskal–Wallis ANOVA for each toxin family to compare the populations, which allowed us to confirm the results from the parametric ANOVA; this latter test requires no assumptions about data distribution [96]. We computed effect

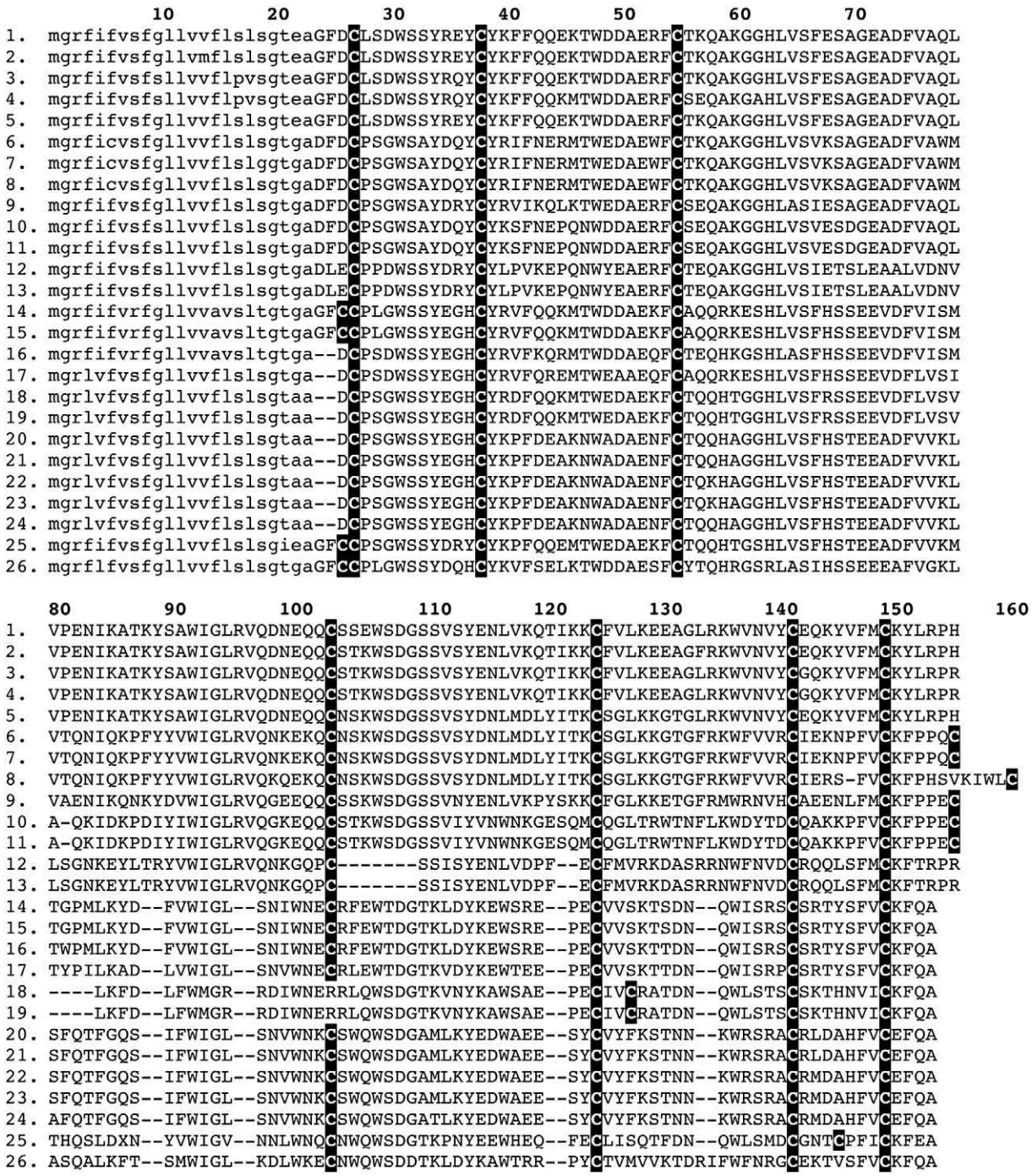

Fig. 3 – Sequence alignment of lectins from *C. o. helleri*: α) 1. GAKQ01000018 CohCI-5, 2. GALC01000015 CohLL-3, 3. GAKQ01000016 CohCI-3, 4. GAKS01000016 CohPH-3, 5. GAKQ01000015 CohCI-2, 6. GAKQ01000014 CohCI-1, 7. GALC01000013 CohLL-1, 8. GAKS01000014 CohPH-1, 9. GALC01000014 CohLL-2, 10. GAKQ01000017 CohCI-4, 11. GAKS01000017 CohPH-4, 12. GALC01000017 CohLL-5, 13. GALC01000016 CohLL-4; and β) 14. GALC01000020 CohLL-3, 15. GALC01000018 CohLL-1, 16. GALC01000022 CohLL-5, 17. GAKS01000021 CohPH-4, 18. GAKS01000022 CohPH-5, 19. GALC01000021 CohLL-4, 20. GAKQ01000022 CohCI-4, 21. GAKQ01000021 CohCI-3, 22. GAKQ01000020 CohCI-2, 23. GAKQ01000019 CohCI-1, 24. GAKS01000019 CohPH-1, 25. GAKS01000020 CohPH-3, 26. GAKS01000023 CohPH-6. CI = Catalina Island, LL = Loma Linda, PH = Phelan. Signal peptide is shown in lowercase, cysteines are highlighted in black.

sizes (approximate variance explained) as adjusted partial eta-squared (η^2) for the parametric ANOVA and as η^2 (computed as χ^2 / [total N – 1]) for the Kruskal–Wallis ANOVAs [96,97]. Eta-squared values ≥ 0.14 are generally deemed large [98]. We conducted these analyses using SPSS 13.0 for Windows, with alpha = 0.05. Following Nakagawa [99], we did not apply Bonferroni adjustments to multiple tests.

3. Results and discussion

Random sequencing recovered sequences for 13 different venom protein encoding gene families (Table 1), with all but Kunitz and Hyaluronidase recovered by both proteomics and transcriptomics. The inability of our combined approach to detect these two venom-components in both result sets may be due to a number of factors, such as, i) differential transcription/translation: not all toxins being replenished at equal stoichiometric rates or simultaneously; ii) technical limitation: the relative separation ability of the HPLC column utilized; iii) co-elution of toxins: one toxin type dominating another and thus obscuring the signal of a toxin present in significantly lower amounts; iv) transcriptomics: the non-exhaustive random sampling procedure utilized which would statistically be likely to recover the most abundant toxin types, with lower-level expressed toxins not recovered; and/or v) microRNA silencing: whereby toxin coding regions undergo transcription but not translation [100]. Lectin toxins, however, were conspicuously absent in both the proteomics and transcriptomics of the Idyllwild population. Sequences analyzed in this study have the GenBank accession numbers of: *C. o. helleri* (Catalina Island) GAKQ01000001–GAKQ01000026; *C. o. helleri* (Idyllwild) GAKR01000001–GAKR01000018; *C. o. helleri* (Loma Linda) GALC01000001–GALC01000026; and *C. o. helleri* (Phelan) GAKS01000001–GAKS01000031. It must be noted that in accordance with the new GenBank deposition rules to exclude fragments of less than 200 base pairs, only the full length sequences were deposited. Thus 27 β-defensins were not deposited, even though their processed and secreted toxin regions were sequenced (only regions of the signal peptide were incomplete). Thus, while these sequences could not be deposited into GenBank, they were utilized in the analyses and are included in the Supplementary material.

Our proteomics analyses revealed significant differences in the venoms of the four populations (Fig. 2), with venom RP-HPLC profiles within a population largely congruent among individuals (Supplementary Fig. 8; note: only two Loma Linda specimens were able to be analyzed due to the rarity of *C. o. helleri* in this locality). The parametric ANOVA yielded a highly significant interaction between population and toxin family ($F_{9.8,22.9} = 13.15$, $P < 0.001$, adjusted partial $\eta^2 = 0.31$; Greenhouse–Geisser adjustment of degrees-of-freedom applied), indicating that the distribution of toxins among the toxin families differed significantly among the populations. The Kruskal–Wallis ANOVAs confirmed that toxin quantity varied significantly among populations for some (nerve growth factor, cysteine-rich secretory protein [CRiSP], lectin; all $P = 0.21$–0.35, $\eta^2 = 0.86$–0.97) but not all toxin families. Five additional toxins (BPP, β-defensin, kallikrein, PLA_2, SVMP) approached significance ($P < 0.10$) with exceptional effect sizes ($\eta^2 > 0.63$). Thus, the ANOVAs confirmed population differences despite the small sample sizes.

Some toxin types were notable for being either highly conserved in their coding sequences (β-defensin, natriuretic), whereas others were extremely variable (kallikrein, lectin, PLA_2, SVMP). While the β-defensins and bradykinin potentiating peptides (BPPs) were of low complexity, our proteomics analyses of the relative expression levels revealed that they are expressed

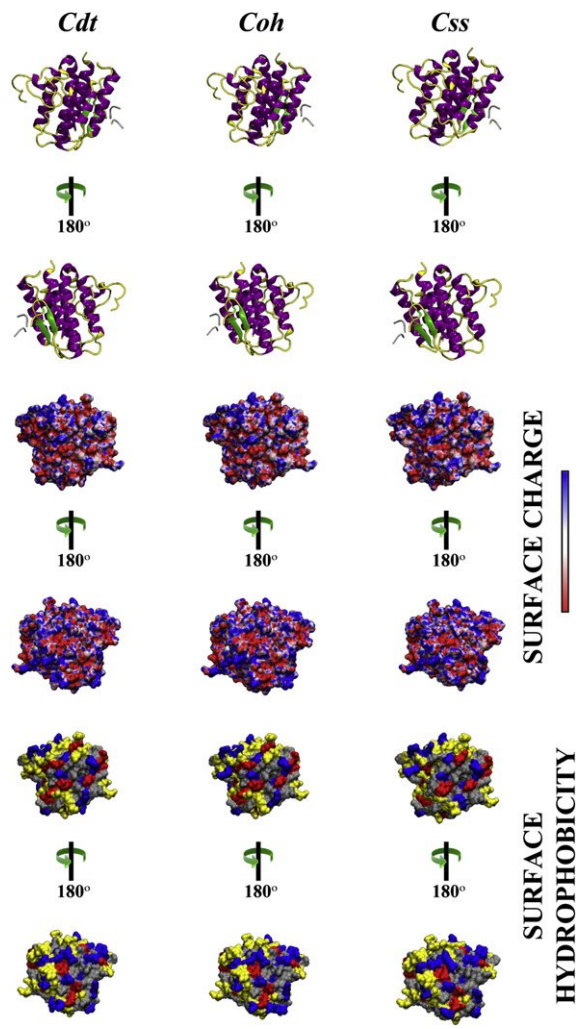

Fig. 4 – Comparative ribbon, surface charge and surface hydrophobicity of the hetrodimeric presynaptic neurotoxic phospholipase A_2 complex from the venoms of *Crotalus durissus terrificus* (Cdt) (PDB: 3R0L; UniProt: P08878 [acidic subunit]; UniProt: P0CG56 [basic subunit]), *C. o. helleri* (Coh) (GenBank: GAKR01000015 [acidic subunit] and GenBank: GAKR01000016 [basic subunit]) and *Crotalus scutulatus scutulatus* (Css) (UniProt: P18998 [acidic subunit] and UniProt: P62023 [basic subunit]). Cartoon images show helices in purple, sheets in green and other structural regions in yellow. Surface charge potentials were mapped on surfaces allowing for color scale data range values of −10.00 to +10.00 using the RWB coloring scheme. Surface residue hydrophicity mapping of residue-type surfaces depicts acidic residues in red, basic residues in blue, polar residues in yellow, and nonpolar residues in silver.

in very high amounts in all populations, with β-defensin in particular invariantly expressed in large quantities (Fig. 2). The multi-product natriuretic/BPP precursor was invariant within and between populations in both the plesiotypic natriuretic peptide domain and the apotypic (derived) BPP domains located within the propeptide region. In contrast, the lectin sequences were highly variable, including the apotyposis of novel cysteines which may facilitate novel structural folding or unique subunit formation with lectins or other toxin types (Fig. 3). Consistent with the proteomic results of this study and a previously published study of San Jacinto Mountain specimens [49] as well as observed notable clinical effects, only the Idyllwild population contained both the acidic and basic subunits of the neurotoxic PLA_2 complex type, with both chains virtually identical to the well-characterized potent presynaptic neurotoxins from *C. d. terrificus* and *C. s. scutulatus* (Fig. 4). It was also notable that the Idyllwild population secreted the lowest amount of SVMPs (Fig. 2), with only a single isoform obtained in the transcriptome and only detectable in trace levels in the proteome. In contrast, the other populations secreted SVMPs in large amounts, with the Phelan having the greatest complexity while the Catalina Island population had less complexity but a much higher relative expression level. This is consistent with the pattern observed for *C. s. scutulatus*, that there is an inverse relationship between the relative amount of neurotoxic PLA_2 and hemorrhagic SVMP [37,101,102]. Thus, it is quite evident how a biochemical arsenal with such variability in neurotoxic, hemotoxic and myotoxic venom-components can complicate clinical treatment of bite victims, not only through the production of highly variable clinical effects, but also as a consequence the reciprocal variability in the efficacy of anti-venom binding. It should be noted that the venom proteomics of

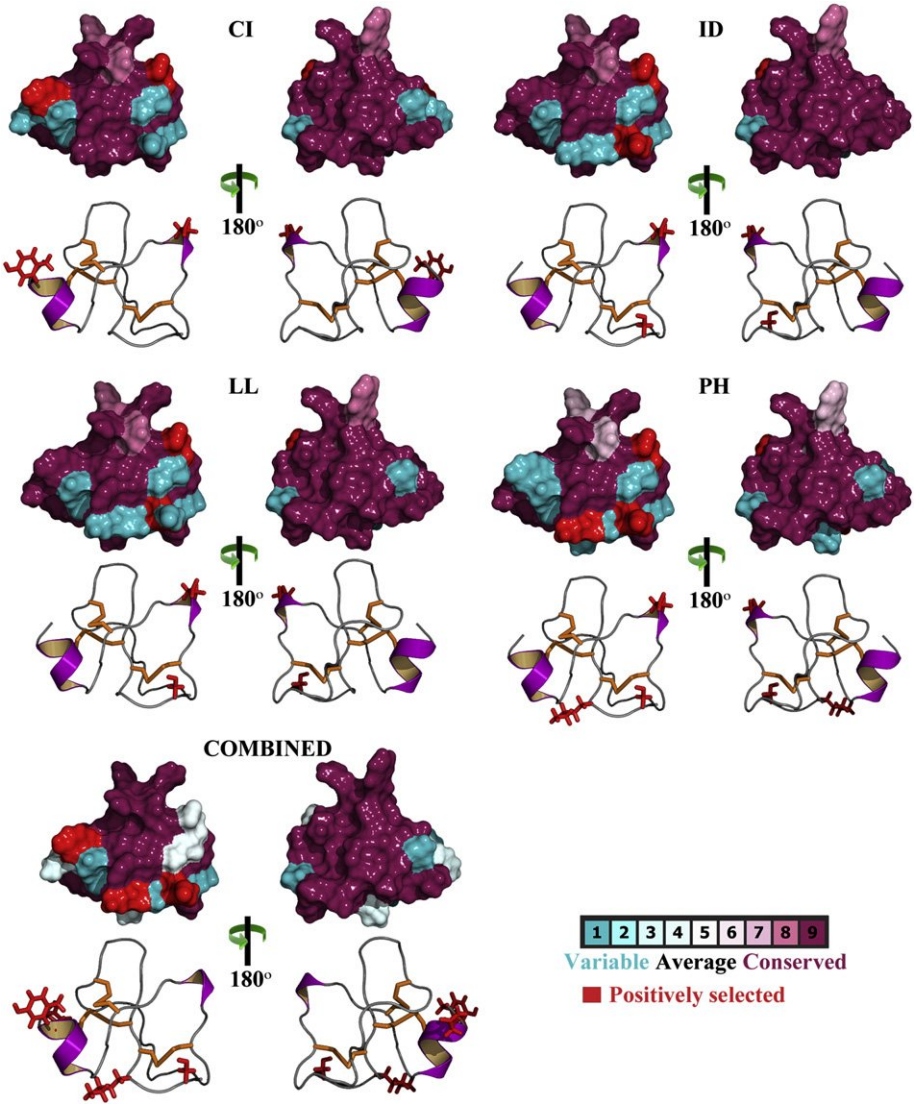

Fig. 5 – Molecular evolution of *C. o. helleri* β-defensins. Three-dimensional homology models (built using the PDB template 1Z99) of β-defensins with evolutionary conservation of amino acids mapped onto them, depicting the locations of positively selected sites (in red) detected by site-model 8 (PP ≥ 0.95, BEB). Schematic representation of the models, which not only depicts the locations of positively selected sites (red sticks) but also highlights disulfide bonds (orange sticks), α helices (purple) and β sheets (green), are also presented.

multiple animals (n = 3; except Loma Linda population, where these animals are extremely rare) from the same region were fairly similar. Hence, it can be safely assumed that the venom-gland transcriptomics of randomly chosen animals represents the overall venomics (genetic makeup of the venom gland) of the representative population.

Understanding the nature and strength of natural selection pressures, which sculpt genetic diversity, is the central theme of molecular evolutionary studies. Since non-synonymous mutations are more likely to influence the structure and function of a protein and hence in turn influence the fitness of the organism, evaluating the rate of accumulation of non-synonymous mutations (dN) in genes, relative to synonymous mutations (dS), as a ratio known as ω (or dN/dS ratio), is essential. We assessed the role of evolutionary selection pressures in shaping various venom proteins in different populations of *C. o. helleri* using various state-of-art selection assessment methodologies. We detected a significant influence of positive Darwinian selection on the evolution of most venom protein encoding genes in these snakes (Figs. 5–7; Tables 2–4; Supplementary Tables 2–5; Supplementary Figs. 1–7 and 9–11).

Site-specific selection assessments indicated that β-defensins, which were expressed in relatively large amounts by all *C. o. helleri* populations examined, followed a regime of weak positive selection: Catalina Island: ω = 1.33 and 3 positively selected (PS); Idyllwild: ω = 2.07 and 3 PS; Loma Linda: ω = 1.14 and 2 PS; Phelan: ω = 1.31 and 5 PS; All: ω = 1.18 and 11 PS (Fig. 5; Table 2). However, the mapping of mutations onto sequence alignments indicated that most hypermutable sites detected by site-specific methods in β-defensins were concentrated in the non-secreted regions of the toxin that are not likely to contribute in the envenoming process. It was also evident

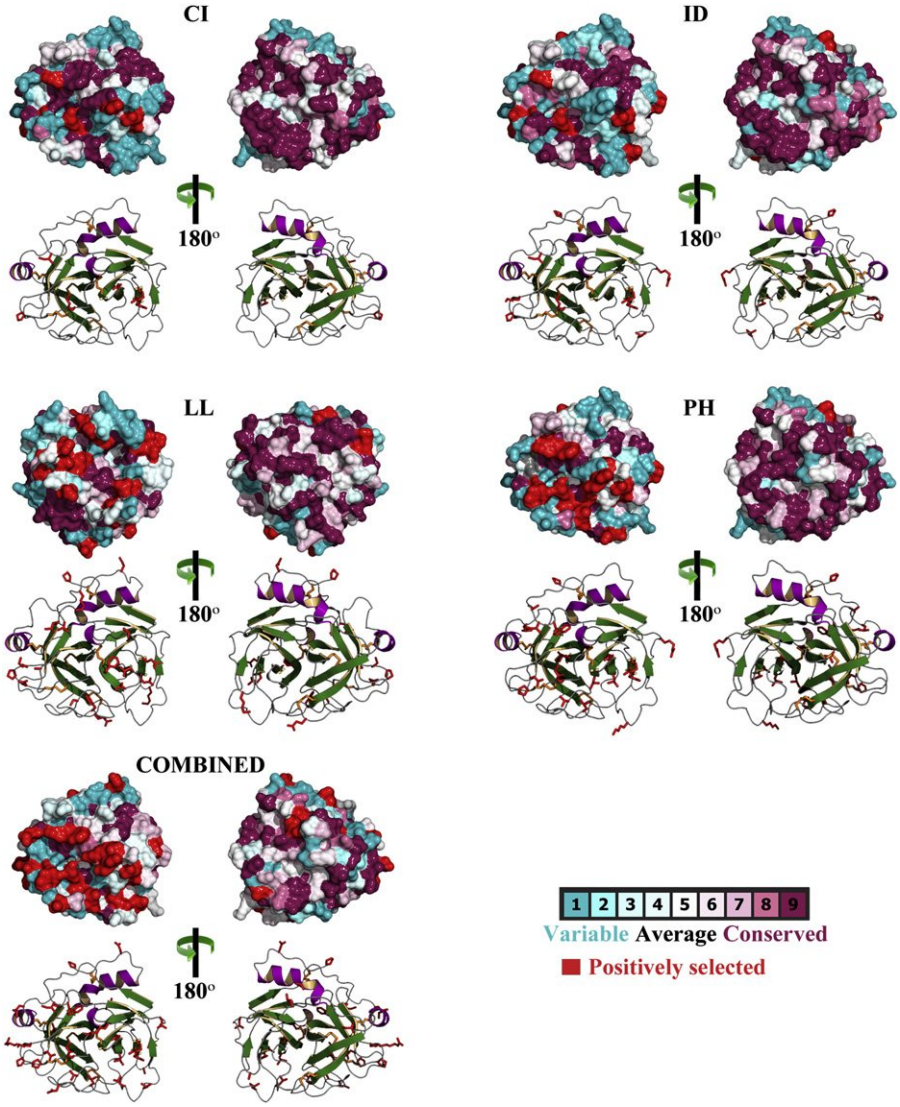

Fig. 6 – Molecular evolution of *C. o. helleri* kallikreins. Three-dimensional homology models (Loma Linda population modeled using the PDB template 1OP0; all others using 2AIQ) of kallikreins with evolutionary conservation of amino acids mapped onto them, depicting the locations of positively selected sites (in red) detected by site-model 8 (PP ≥ 0.95, BEB) are presented. Schematic representation of the models, which not only depicts the locations of positively selected sites (red sticks) but also highlights disulfide bonds (orange sticks), α helices (purple) and β sheets (green), are also presented.

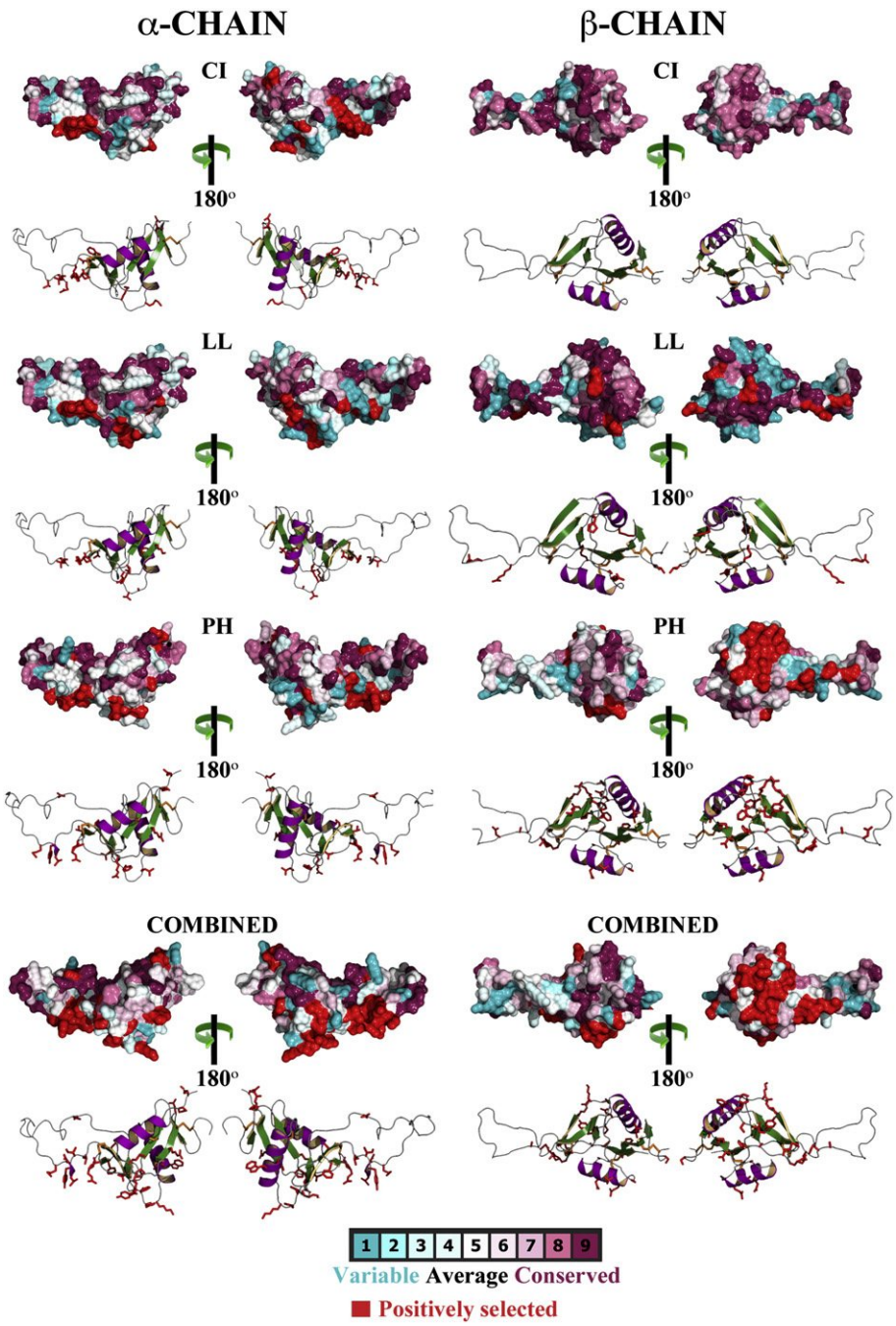

Fig. 7 – Molecular evolution of *C. o. helleri* lectin α- and β-chains. Three-dimensional homology models (α-chain lectins: Catalina Island population and the 'combined set' modeled using PDB template 1C3A; others using 1UMR; β-chain lectins: Loma Linda population modeled using the template 1V4L; all others using 1J34) of lectin α- and β-chains with evolutionary conservation of amino acids mapped onto them, depicting the locations of positively selected sites (in red) detected by site-model 8 (PP ≥ 0.95, BEB). Schematic representation of the models, which not only depicts the locations of positively selected sites (red sticks) but also highlights disulfide bonds (orange sticks), α helices (purple) and β sheets (green), are also presented.

that the entire stretch of nucleotides encoding the secreted region of β-defensins evolved under the extreme influence of negative selection, with 76% of residues being extremely well conserved (percent identity ≥ 90%; Supplementary Fig. 1). This was also supported by the results of MEME, an extremely accurate method of detecting episodic bursts of adaptation, which detected fewer episodically diversifying sites in β-defensins (Table 2). Mapping of variable sites on the structure of the β-defensin 'crotamine' from *C. d. terrificus* (PDB code: 1Z99 [103]), which is homologous and thus structurally very similar

Table 2 – *C. o. helleri* intraspecific venom dynamics: β-defensins.

Population	FUBAR [a]	MEME sites [b]	BSR [c]	PAML [d]	
				M8	M2a
CI	ω > 1 [e]: 0 ω < 1 [f]: 1	0	1	3 (2 + 1) 1.33	3 (2 + 1) 1.33
ID	ω > 1 [e]: 1 ω < 1 [f]: 0	0	2	3 (2 + 1) 2.07	3 (2 + 1) 2.07
LL	ω > 1 [e]: 0 ω < 1 [f]: 0	1	1	2 (0 + 2) 1.14	0 1.14
PH	ω > 1 [e]: 1 ω < 1 [f]: 1	0	4	5 (1 + 4) 1.31	2 (1 + 1) 1.31
Combined	ω > 1 [e]: 0 ω < 1 [f]: 0	4	6	11 (5 + 6) 1.18	6 (3 + 3) 1.16

ω: mean dN/dS.
Populations: CI = Catalina Island; ID = Idyllwild; LL = Loma Linda; PH = Phelan.
[a] Fast Unconstrained Bayesian AppRoximation.
[b] Sites detected as experiencing episodic diversifying selection (0.05 significance) by the Mixed Effects Model Evolution (MEME).
[c] Number of branches detected by the branch-site REL (random effects likelihood) test as episodically diversifying.
[d] Positively selected sites detected by the Bayes Empirical Bayes approach implemented in M8 and M2a. Sites detected at 0.99 and 0.95 significance are indicated in the parenthesis.
[e] Number of sites under pervasive diversifying selection at the posterior probability ≥ 0.9 (FUBAR).
[f] Number of sites under pervasive purifying selection at the posterior probability ≥ 0.9 (FUBAR).

Table 3 – *C. o. helleri* intraspecific venom dynamics: Kallikrein.

Population	FUBAR [a]	MEME sites [b]	BSR [c]	PAML [d]	
				M8	M2a
CI	ω > 1 [e]: 8 ω < 1 [f]: 5	6	4	7 (6 + 1) 1.35	7 (4 + 3) 1.31
ID	ω > 1 [e]: 20 ω < 1 [f]: 4	6	6	11 (2 + 9) 1.63	11 (2 + 9) 1.63
LL	ω > 1 [e]: 23 ω < 1 [f]: 8	9	9	24 (8 + 16) 1.60	15 (7 + 8) 1.57
PH	ω > 1 [e]: 22 ω < 1 [f]: 7	15	5	24 (10 + 14) 1.38	15 (7 + 8) 1.41
Combined	ω > 1 [e]: 27 ω < 1 [f]: 14	45	12	36 (18 + 18) 1.36	25 (13 + 11) 1.38

ω: mean dN/dS.
Populations: CI = Catalina Island; ID = Idyllwild; LL = Loma Linda; PH = Phelan.
[a] Fast Unconstrained Bayesian AppRoximation.
[b] Sites detected as experiencing episodic diversifying selection (0.05 significance) by the Mixed Effects Model Evolution (MEME).
[c] Number of branches detected by the branch-site REL (random effects likelihood) test as episodically diversifying.
[d] Positively selected sites detected by the Bayes Empirical Bayes approach implemented in M8 and M2a. Sites detected at 0.99 and 0.95 significance are indicated in the parenthesis.
[e] Number of sites under pervasive diversifying selection at the posterior probability ≥ 0.9 (FUBAR).
[f] Number of sites under pervasive purifying selection at the posterior probability ≥ 0.9 (FUBAR).

to β-defensin, revealed that the N-terminal positions 23 (Y in sequence 1; Supplementary Fig. 1) and 25 (R in sequence 1; Supplementary Fig. 1) as well as the C-terminal residues in position 61 (K in sequence 1; Supplementary Fig. 1), 62 (S in sequence 1; Supplementary Fig. 1), and 63 (G in sequence 1; Supplementary Fig. 1) are structurally more flexible (for the remaining positively selected amino acids 3D-structure coordinates were not resolved). This can be explained by the fact that these locations in the protein structure fall outside the disulfide bond-stabilized core. Although the highly conserved positions, such as 28 (K), 53 (R), 54 (W), and 55 (R; all referred to in sequence 1; Supplementary Fig. 1) were also solvent exposed, they were located inside the disulfide bridge-stabilized protein core and thus experienced heavy constraints of negative selection. However, the lack of variation in secreted regions of β-defensins may be indicative of their unique mode of action as peptides non-specifically target and destabilize the negatively charged microbial membranes using their cationic amino acid residues, resulting in membrane permeabilization [104]. Not-surprisingly, 29% of the residues in *C. o. helleri* β-defensins were cationic (K, R and H) and were extremely well conserved (percent identity ≥ 80%; Supplementary Fig. 1). Hence, it is expected that the evolutionary constraints favor the preservation of cationic residues required for toxicity. The branch-site REL (BSR) test, which significantly identifies lineages that follow the regime of episodic diversification, clearly highlighted the differences in strengths of evolutionary selection pressures acting upon β-defensins in *C. o. helleri* populations (Supplementary Fig. 5). In the Phelan population this test detected as many as four episodically diversifying branches in β-defensin gene lineage, while detecting only one branch each in Catalina Island and Loma Linda populations, and two branches in the Idyllwild population (Supplementary Fig. 5).

While the kallikreins found in each of the *C. o. helleri* populations examined were found to be rapidly evolving under the influence of positive selection [Catalina Island: ω = 1.35 and 7 PS; Idyllwild: ω = 1.63 and 11 PS; Loma Linda: ω = 1.60 and 24 PS; Phelan: ω = 1.38 and 24 PS; All: ω = 1.36 and 36 PS] (Fig. 6; Supplementary Fig. 2), the number of positively selected sites detected by M8's Bayes empirical Bayes (BEB) approach, varied from 7 to 24, highlighting the differential rate of evolution of kallikreins in various *C. o. helleri* populations (Table 3). The number of branches detected by the BSR test as episodically diversifying in kallikrein encoding genes varied from 4 to 10 in various populations (Supplementary Fig. 6), again highlighting the differential role of selection in shaping these venom protein encoding genes.

Lectin α-chain [Catalina Island: ω = 2.36 and 15 PS; Loma Linda: ω = 2.51 and 14 PS; Phelan: ω = 3.07 and 16 PS; All: ω = 2.23 and 27 PS] and lectin β-chain [Catalina Island: ω = 0.46 and 0 PS; Loma Linda: ω = 2.73 and 11 PS; Phelan: ω = 2.34 and 28 PS; All: ω = 2.29 and 29 PS] were found to evolve under the significant

Table 4 – *C. o. helleri* intraspecific venom dynamics: Lectins.

Population	FUBAR [a]	MEME sites [b]	BSR [c]	PAML [d]	
				M8	M2a
α chain					
CI	ω > 1 [e]: 12 ω < 1 [f]: 5	2	4	15 (8 + 7) 2.36	12 (5 + 7) 2.34
LL	ω > 1 [e]: 15 ω < 1 [f]: 2	1	3	14 (7 + 7) 2.51	12 (5 + 7) 2.51
PH	ω > 1 [e]: 15 ω < 1 [f]: 4	0	4	16 (8 + 8)	13 (7 + 6)
Combined	ω > 1 [e]: 26 ω < 1 [f]: 6	7	7	27 (14 + 13) 2.23	23 (14 + 9) 2.24
β chain					
CI	ω > 1 [e]: 0 ω < 1 [f]: 1	0	0	0 0.46	0 0.46
LL	ω > 1 [e]: 2 ω < 1 [f]: 0	0	1	11 (0 + 11) 2.73	2 (0 + 2) 2.73
PH	ω > 1 [e]: 16 ω < 1 [f]: 2	5	6	28 (12 + 16) 2.34	22 (9 + 13) 2.34
Combined	ω > 1 [e]: 22 ω < 1 [f]: 2	5	6	29 (16 + 13) 2.29	20 (11 + 9) 2.31

ω: mean dN/dS.
Populations: CI = Catalina Island; ID = Idyllwild; LL = Loma Linda; PH = Phelan.
[a] Fast Unconstrained Bayesian AppRoximation.
[b] Sites detected as experiencing episodic diversifying selection (0.05 significance) by the Mixed Effects Model Evolution (MEME).
[c] Number of branches detected by the branch-site REL (random effects likelihood) test as episodically diversifying.
[d] Positively selected sites detected by the Bayes Empirical Bayes approach implemented in M8 and M2a. Sites detected at 0.99 and 0.95 significance are indicated in the parenthesis.
[e] Number of sites under pervasive diversifying selection at the posterior probability ≥ 0.9 (FUBAR).
[f] Number of sites under pervasive purifying selection at the posterior probability ≥ 0.9 (FUBAR).

influence of positive selection (Table 4; Supplementary Fig. 3). However, β-chain of the Catalina Island population was remarkably revealed to have evolved under the influence of negative selection (ω = 0.46, 0 PS; Table 4; Supplementary Fig. 4). Other than at position 56, amino acid residues in all other positions were invariant (Supplementary Fig. 4). The rapid rate of molecular evolution observed in lectins is consistent with the great diversity of novel sequences recovered, including the apotyposis or the derivation of novel cysteine residues (Fig. 3). The rapid accumulation of hypermutable sites under the influence of positive selection in β-chain lectins from all *C. o. helleri* populations except those from Catalina Island, where the toxin-encoding gene has evolved under strong negative selection, is intriguing and warrants further experimental evaluations to understand the stark differences in the magnitude of selection pressures. While the BSR test detected a few lineages as episodically diversifying in the α-chain lectins of various populations, the results of this test in the β-chain lectins were particularly interesting (Supplementary Fig. 7). This test failed to detect any branch in the Catalina Island population, while detecting a single branch in Loma Linda population as episodically diversifying (Supplementary Fig. 7). In contrast, as many as 6 branches were detected as following the regime of episodic adaptation in β-chain lectins of the Phelan population (Supplementary Fig. 7). Similar to the results of all state-of-art selection assessment methods outlined above, the evolutionary fingerprints of venom-encoding genes in *C. o. helleri* clearly depicted the differential influence of natural selection on their evolution (Supplementary Figs. 9–11).

The structure and surface chemistry of the presynaptic PLA_2 complex from *C. o. helleri* is very well conserved when compared to the homologues from *C. d. terrificus* and *C. s. scutulatus* (Fig. 4). Both amino acid type distribution on the protein surface as well as studying surface charges and surface hydrophobicity of all three PLA_2 complexes revealed only minor differences. While the positive and negative charged patches in globo were located in the same positions, minimal differences were observed in the size and charge of these surface regions. Since the PLA_2s of *C. d. terrificus* and *C. s. scutulatus* are well-characterized to be potent neurotoxins (cf. [89,105]), we conclude that the described similarities of *C. o. helleri* PLA_2 to the former ones are responsible for neurotoxic effects of PLA_2s observed in the *C. o. helleri* population. The precise evolutionary regimes followed by genes encoding PLA_2 and SVMPs in these snakes remain to be elucidated.

Thus, it is evident that *C. o. helleri* venom-encoding genes have experienced differential evolutionary selection pressures. Differential rate of molecular evolution or expression occurred not only between toxin types within the venom of a particular population, but also for the same toxin type between populations. These results demonstrate that the different populations of *C. o. helleri* follow distinct evolutionary trajectories, with the differential venom profiles likely driven by variation in predatory ecology. This is a reflection of the complex evolutionary history of this species, which ranges from sea level to high mountain peaks and occupies a diverse range of habitats. These habitats possess differing lizard and mammal prey assemblages [106–108], and evidence from other snakes suggests that strong natural selection has driven venom adaptation to particular prey species [12,20,21,30,31,40,46,47,59,61–63,109]. Although climate might be expected to influence venom composition, our data suggest otherwise concerning the dichotomy of type I (proteolytic or "tenderizer") versus type II (more toxic) venoms [70]. It has been suggested that snakes at higher elevation with the greatest temperature fluctuations could be expected to possess a type I venom to facilitate digestion [70]. However, the population that faces the highest temperature fluctuations (Idyllwild) possesses a type II venom that lacks almost entirely the metalloproteases typical of type I venoms. These results also indicate significant differences in potential human envenomation profiles, consistent with the complex clinical picture previously observed, with some populations being hemorrhagic while others are neurotoxic. The exquisite diversity of venom-components highlighted in this study and the variation in intensity and the nature of natural selection shaping the molecular toxin scaffolds may not only result in distinct envenoming profiles but may also induce variable responses to antivenom. Hence, understanding the true molecular diversity of venom and the evolutionary forces that shape them not only

aids in the prediction of clinical effects but also reveals that these highly variable venoms are a rich source of novel toxins, some of which may have significant potential for use as lead compounds in drug design and development. Thus, the results of this study not only contribute to the body of knowledge regarding venom evolution but also have applied outcomes both from a clinical perspective and also from drug design. These results will also be useful in science communication to demonstrate that there is indeed significant variation in the venom of this medically important species, but that such evolution has not occurred recently but rather the venom diversity seen today is reflective of the long evolutionary history, not of recent changes as popularly misunderstood. Thus this species is a model for the broader penetration of lay-person understanding of venom diversity and the clinical and economic importance of such variation.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jprot.2014.01.013.

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Tracking Number: 2015-006 ~~(Click here to enter text.)~~

To request a change to regulations under the authority of the California Fish and Game Commission (Commission), you are required to submit this completed form to: California Fish and Game Commission, 1416 Ninth Street, Suite 1320, Sacramento, CA 95814 or via email to FGC@fgc.ca.gov. Note: This form is not intended for listing petitions for threatened or endangered species (see Section 670.1 of Title 14).

Incomplete forms will not be accepted. A petition is incomplete if it is not submitted on this form or fails to contain necessary information in each of the required categories listed on this form (Section I). A petition will be rejected if it does not pertain to issues under the Commission's authority. A petition may be denied if any petition requesting a functionally equivalent regulation change was considered within the previous 12 months and no information or data is being submitted beyond what was previously submitted. If you need help with this form, please contact Commission staff at (916) 653-4899 or FGC@fgc.ca.gov.

SECTION I: Required Information.

Please be succinct. Responses for Section I should not exceed five pages

1. **Person or organization requesting the change (Required)**
   Name of primary contact person: Dennis Thibeault, Vice President Forestry, Mendocino Redwood Company, LLC
   Address: [redacted]
   Telephone number: [redacted]
   Email address: [redacted]

2. **Rulemaking Authority (Required) -** Reference to the statutory or constitutional authority of the Commission to take the action requested: California Fish & Game Code § 1580; California Fish & Game Code § 2855; California Public Resources Code § 36600, 36700 , 36725[(a),(e)]

3. **Overview (Required) -** Summarize the proposed changes to regulations: Remove special closure regulations for Rockport Rocks in 14 CCR § 632 (b)(17)

4. **Rationale (Required) -** Describe the problem and the reason for the proposed change: The above-mentioned special closure was enacted on a parcel private property owned by Mendocino Redwood Company, LLC (MRC). The special closure as currently written prohibits complete access to this parcel of land from March 1 to August 31. MRC was never informed—neither verbally nor in writing—by the California Department of Fish and Wildlife or the North Coast Regional Stakeholders Group about including Rockport Rocks in a special closure when formally proposed in 2010. Evidence from historical documents establishing the North Coast Marine Protected Area (MPA) indicate that the designation of Rockport Rocks as a special closure area was an unintentional error because it was mistakenly assumed to be a part of the Coastal National Monument managed by the Bureau of Land Management. The CDFW is also on record stating that MPAs "will not affect private property rights" and that the "MPA designation process must take into account existing California State Lands Commission leases, California Fish and Game Commission state water bottom and kelp leases, tide and submerged lands grants, private tidelands, and any other legal entitlements." Overall, had these facts been disclosed during the MLPA process, this area would have been removed from the original proposal prior to the Commission's vote on the matter.

SECTION II: Optional Information

5. Date of Petition: November 17, 2015

6. Category of Proposed Change

☐ Sport Fishing
☐ Commercial Fishing
☐ Hunting
☒ Other, please specify: Special Closure Area for

7. The proposal is to: (*To determine section number(s), see current year regulation booklet or https://govt.westlaw.com/calregs*)

☐ Amend Title 14 Section(s):Click here to enter text.
☐ Add New Title 14 Section(s): Click here to enter text.
☒ Repeal Title 14 Section(s): 14 CCR § 632 (b)(17)

8. If the proposal is related to a previously submitted petition that was rejected, specify the tracking number of the previously submitted petition Click here to enter text.
Or ☒ Not applicable.

9. Effective date: If applicable, identify the desired effective date of the regulation.
If the proposed change requires immediate implementation, explain the nature of the emergency: As soon as possible; the designation of Rockport Rocks special closure area was an unintentional error as all the facts of ownership were neither made available to the CDFW nor the Commission during the special closure designation process.

10. Supporting documentation: Identify and attach to the petition any information supporting the proposal including data, reports and other documents: Supplementary information including a cover letter substantiating MRC's case is attached to this petition.

11. Economic or Fiscal Impacts: Identify any known impacts of the proposed regulation change on revenues to the California Department of Fish and Wildlife, individuals, businesses, jobs, other state agencies, local agencies, schools, or housing: Designation of Rockport Rocks as a special closure is a potential encumbrance to MRC being able to sell the parcel or sell a conservation easement to an interested party.

12. Forms: If applicable, list any forms to be created, amended or repealed:

Click here to enter text.

SECTION 3: FGC Staff Only

Date received: Click here to enter text.

FGC staff action:

☒ Accept - complete

☐ Reject - incomplete
☐ Reject - outside scope of FGC authority

Tracking Number

Date petitioner was notified of receipt of petition and pending action: 11/24/15

Meeting date for FGC consideration: Receive 12/9/15, Action 2/10/16

FGC action:

☐ Denied by FGC
☐ Denied - same as petition _____

Tracking Number

☐ Granted for consideration of regulation change

Allweather Wood™ Humboldt Redwood™ MENDOCINO

November 23, 2015

Mr. Jack Baylis, President
California Fish and Game Commission
1416 Ninth Street, Suite 1320
Sacramento, CA 95814

Dear Mr. Baylis

We have recently been made aware a parcel of our property, referred to as "Rockport Rocks," was included in a special closure area during the Marine Life Protection Act (MLPA) designation process. Unfortunately this occurred without any type of notification to us from the California Department of Fish and Wildlife (CDFW) or the North Coast Regional Stakeholders Group (NCRSG). MRC's ownership of Rockport Rocks (aka "Sea Lion Rock") is well-established by a patent, grant deed, and numerous historical documents and photographs spanning nearly a century and are available upon request.

This special closure, 14 CCR § 632(b)(17), inhibits our private property rights and our ability to enjoy our property. We provide limited public access to Rockport Beach and the beach is visited and enjoyed by hundreds of employees, family and friends every year. The seasonal closure, which goes from March 1 to August 31, effectively prohibits access to this parcel of land by the property owner, and potentially limits recreational and educational activities (e.g., fishing, abalone diving, kayaking, kelp harvesting, bird watching, tidepooling, etc.) in nearshore waters historically enjoyed by visitors to Rockport Beach.

The public nature of the special closure has also created a potential encumbrance to MRC's ability to sell the parcel or negotiate a conservation easement with an interested party should it ever decide to do so. In 2011, the Bureau of Land Management (BLM) and the United States Fish and Wildlife Service indicated interest in acquiring Rockport Rocks for conservation purposes in the Report "Potential Murre Restoration Projects Northern California".

Our investigation into this matter leads us to conclude that the inclusion of MRC property in a special closure was done in error. First, documents for the MLPA process suggest that the CDFW and the NCRSG assumed that Rockport Rocks were part of the publically owned California Coastal National Monument system administered by the BLM. Secondly, the CDFW stated in a memorandum dated 1/31/08 from John Ugoretz to the MLPA Stakeholder Group that MPAs "will not affect private property rights" and that the "MPA designation process must take into account existing California State Lands Commission leases, California Fish and Game Commission state water bottom and kelp leases, tide and submerged lands grants, private tidelands, and any other legal entitlements". In fact, the Vizcaino Rocks special closure, located 0.6 miles to the south of Rockport Rocks, clearly embodies this philosophy as it has a modified boundary that extends 300' from only the seaward side of the rock, presumably because a buffer around the entire rock would overlap with a private beach owned by the Save-the-Redwoods League.

We conclude had CDFW, NCRSG, or the Commission known MRC was the owner of Rockport Rocks, this special closure would not have been included in the final rulemaking package that was eventually adopted. Based on the facts presented here, we kindly request that the Commission remove the special closure regulations on Rockport Rocks.

If you have any questions, would like documentation of the above statements or would like to discuss the matter further, please give me a call at (707) 463-5112 or email me at dthibeault@mendoco.com.

Sincerely,

Dennis Thibeault
Vice President Forestry

ABOUT MRC

Mendocino Redwood Company (MRC) was created in 1998 from lands purchased in Mendocino and Sonoma counties with the publicly declared mission to be good stewards of the forest and at the same time run a successful business. We have made significant progress in that regard:

1. *Adopting policies to make MRCs forestlands FSC certified (since November 2000);*
2. *Adding more than 1 billion board feet of redwood and Douglas fir trees by lowering the rate of harvest;*
3. *Defining of old growth down to the level of an individual tree, along with implementation of a policy to protect all individual old growth trees across our property;*
4. *Elimination of traditional clear cutting from our property;*
5. *Long term investments to improve habitat for fish across the property by controlling or holding back more than 1 million cubic yards of sediment (more than 100,000 dump trucks of dirt) from the coastal streams flowing through our forest;*
6. *Removal of more than 36 long time fish barriers, increasing fish bearing streams by more than 20 miles.*
7. *Operating as an open and transparent business; including an open invitation to take interested individuals anywhere in the forest;*
8. *Completing a substantial rebuild of our Ukiah sawmill, assuring that Mendocino County will have infrastructure in the processing of wood products for many years to come; and*
9. *Employing about 300 skilled employees in Mendocino County earning family-level wages and benefits.*

From:	Kerry Kriger
To:	Sonke Mastrup; FGC@fgc.ca.gov
Cc:	Chuck Bonham; John Laird
Subject:	DFW bullfrog policies - please respond
Date:	Sunday, November 01, 2015 11:08:02 AM

Dear Sonke Mastrup and the Fish & Game Commission of California,
I sent the letter pasted below to Director Bonham and Secretary Laird on August 26th but have not received a response. I would appreciate a response as soon as possible.
Thank you,

Dr. Kerry Kriger
SAVE THE FROGS!
Founder, Executive Director, Ecologist & California Taxpayer
www.savethefrogs.com
www.savethefrogs.com/kerry-kriger
[redacted]

SAVE THE FROGS! is the world's leading amphibian conservation organization. We work in California, across the USA, and around the world to prevent the extinction of amphibians, and to create a better planet for humans and wildlife. Since 2008, SAVE THE FROGS! has organized over 1,600 educational events in 60 countries to raise awareness for endangered amphibians.

##########################

Dear John and Chuck,

DFW's Betsy Bolster informs us that the DFW only has one total employee dedicated to amphibians and reptiles, and that this is causing continued delays in releasing the DFW's amphibian conservation strategy report "A Guiding Vision for the Conservation and Management of California's Reptiles and Amphibians", which I believe has been in production for four years, an inordinately long time for a report. Given that amphibians and reptiles comprise two out of the five classes of vertebrates in the state, can you please immediately authorize additional staff to (1) complete the above mentioned document and (2) to develop a strategy for the DFW to discontinue issuing permits for the importation of bullfrogs or at the very least to charge importers a high fee for permits, which would deter applications and also fund DFW so that the Department could conduct disease testing of incoming amphibians and enforce regulations related to these infected amphibians.

On a related note, at its February meeting the FGC instructed the DFW to report back to the FGC on its progress related to dealing with the state's bullfrog importation problems but to the best of my knowledge the DFW has not followed through. I would appreciate if you could tell me when the DFW plans to report back on this issue.

Thank you!
Kerry

Coastside Fishing Club
P.O. Box 5501
San Mateo, CA 94402

November 19, 2015

Jack Baylis, President
California Fish and Game Commission
1416 Ninth Street, Suite 1320
Sacramento, CA 95814

SUBJECT: Request for December Agenda Item on Crab Closure

Dear President Baylis:

On behalf of the recreational fishing community, we ask that the Fish and Game Commission's December 9 meeting include a specific agenda item to discuss the ongoing closures of the recreational Dungeness and rock crab fisheries.

On November 7, 2015, the Commission took emergency regulatory action to delay the opening of the recreational Dungeness crab season and to close the rock crab season throughout the State. The Commissioners noted the gravity of this emergency action and assured stakeholders that the fishery would reopen as soon as it is safe. The Department of Fish and Wildlife committed to weekly testing at each port.

Given the extraordinary nature of this action and the continued closures, there is a genuine need for the Commission and the Department to review the status of the closure and the results of the weekly tests at each port. We are aware that the commercial fishing sector strongly objects to the county-by-county regulatory approach adopted by the Commission for recreational anglers, preferring to delay the recreational opener and tie it to a statewide commercial opening date.

Respectfully submitted.

Marc Gorelnik
Coastside Fishing Club

From:	Tom Handley
To:	FGC
Subject:	Petition BCD-001
Date:	Thursday, October 15, 2015 9:03:41 AM

Greetings,

I am writing in regards to the petition submitted by Curtis Haney, tracking number (BCD-001) and am requesting amending Title 14 Section(s):Chapter 4, Section 29.05 (b) (1)
to include the public harvesting of Goose Neck Barnacles for personal consumption. The harvesting of these mollusks pose not more threat to the environment than the already allowed harvesting of mussels. The barnacles are tasty, nutritious, and as far as I know have a healthy population whereby harvesting for personal consumption would not threaten the species's survival.

Thank You,
Tom Handley
Berkeley, CA

From:	Polly Shaw
To:	FGC
Subject:	SUPPORT FOR PETITION SUBMITTED BY CURTIS HANEY, TRACKING NUMBER (BCD-001)
Date:	Friday, October 16, 2015 2:56:05 PM

October 16, 2015

Fish & Game Commission
1416 Ninth St.
Room 1320
Sacramento, CA 95814

RE: SUPPORT FOR PETITION SUBMITTED BY CURTIS HANEY, TRACKING NUMBER (BCD-001) REQUESTING THE AMENDING OF TITLE 14 SECTION(S):CHAPTER 4, SECTION 29.05 (b) (1)

To the Fish and Game Commission;

I am an avid, law-abiding foodie who asks you to change the rules to allow the harvest Goose Neck Barnacles for personal consumption. It's illogical that harvesting them is prohibited, and the decision seems to be based on no science or facts.

Simply put, they're delicious and numerous. And they are hard as heck to harvest, so it's very difficult to pick very many and risk hurting the population. They are as abundant and self-replenishing as mussels. They should be held to the same seasonal and weight harvest rules as mussels, since they're no different.

I have unwittingly been harvesting them once a year at Thanksgiving, without realizing it was prohibited. It makes for a lovely activity and a real present to myself for dinner.

I thoroughly support the petition of Curtis Haney, tracking number BCD-001, requesting the amending of Title 14 Sections: Chapter 4, Section 29.05(b)(1).

Please lift the ban on harvesting Gooseneck Barnacles. Thank you for your consideration.

Polly Shaw
[redacted]

From:	Roy Coto
To:	FGC
Cc:	
Subject:	PETETION TO CHANGE A CURRENT FISH AND GAME COMMISSION REGULATION
Date:	Monday, October 19, 2015 7:41:09 AM

Sirs
I understand that on December 9-10, 2015 the petition submitted by Curtis Haney, tracking number (BCD-001) requesting the amending of Title 14 Section(s):Chapter 4, Section 29.05 (b) (1)
will up for consideration. I would urge you to accept this petition and amend the regulation on goose neck barnacles.

Roy Coto

From:	David Le
To:	FGC
Subject:	Please allow harvesting of gooseneck barnacles
Date:	Sunday, November 22, 2015 2:36:20 PM

Dear Fish and Game,

I am writing to add my support to the petition to allow for the harvesting of gooseneck barnacles.
These animals grow in great abundance along our coastline. The limited harvesting by private individuals would do little to impact the overall population.

Thank you,
David

From: Jean Lundeen
To: FGC
Subject: Support for petition to allow harvesting of goose neck barnacles
Date: Sunday, November 22, 2015 12:08:20 PM

Hello

I support the petition submitted by Curtis Haney, tracking number (BCD-001) requesting the amending of Title 14 Section(s):Chapter 4, Section 29.05 (b) (1)

Please change the regulation so that the public can harvest Goose Neck Barnacles for personal consumption. I understand these are very delicious and would enjoy the opportunity to harvest some. I understand they are not threatened so I hope this can be done.

Thank you,

Jean Lundeen

From:	Carol Reed
To:	FGC
Subject:	amending Title 14 sec. Chap 4, sec29.05(b)(1)
Date:	Saturday, November 21, 2015 11:21:32 PM

Dear Fish and Game Commission,

I writing to you to request a revision on the rule regarding the personal harvesting of (Gooseneck), Leaf Barnacles. They are considered a delicacy in Spain and are allowed to be harvested in Alaska. We previously collected then and they are delicious, a member of the lobster family. Harvesting times are limited to low tides. We previously collected them when we gathered mussels. Presently I have seen more barnacles then muscles in some locations.
Please add them back into the recreational take species.

Please consider the petition submitted by Curtis Haney, tracking number (BCD-001) requesting the amendment of Title 14 Section(s):Chapter 4, Section 29.05 (b) (1)

Thank you,
Carol Reed
Diver, fisher person and supporter of sustainable harvests.

From: Pat Grady
To: FGC
Subject: Roosevelt Elk in Del Norte Cty, CA
Date: Friday, November 13, 2015 2:46:05 PM

I am writing in regard to the future management of the Roosevelt Elk that are located in Del Norte County, CA. I know that many local people delight in seeing the elk in various places along the highway; it is one of the things that makes living here so special. There are many who do not approve of the lottery, but people don't know where to turn to make their voices heard. Also, the elk are one of the unique elements that draw tourists to our area; we should be celebrating them, not killing the finest surviving examples of these unique creatures so someone can hang another head on their wall. I have never had a problem with people legally hunting for 'groceries', but to hold a lottery to win a chance to kill – not cull – the elk who now grace our county lands is reprehensible and reflects poor stewardship on the part of all entities responsible.

I was appalled by the front page local news article where someone proudly spoke of the 'trophy' bull he had hunted through the lottery. Responsible management of the herd(s), in my opinion, means culling only weaker members and females when needed, NOT stripping an already small gene pool of the superior genes you want passed on: that is not culling, that is a lottery for killing. I strongly suggest that you do some serious surveys and counts before hunting of any of the elk at any time. How else can you determine what are sustainable and responsible hunting practices? This needs to be done first! Officials admit that they have no idea how many elk are actually here, but they assume that there are "plenty of elk" for people to kill for no good reason.

I also believe that you need to determine ways to help maintain and improve the genetic integrity of the herds; to do so, the state must work to create wildlife corridors so the elk can continue their natural movements without being slaughtered as they move from public lands through private lands where they are hunted without regard to the future or the best interest of the elk or county. There also need to be corridors that allow the elk to travel to increase genetic diversity. It is not so long since they were virtually extinct – we should be working to improve the limited gene pool that remains, not to decimate it further. Wildlife corridors are being recognized as an important tool for conservation throughout our country; we need to do this for this signature species at least.

I hope that all officials involved will seriously consider viewing the elk as a precious resource – not of some lottery dollars – but as an important part of the biological diversity and great overall natural value of our area. The elk are part and parcel of the incredible ecosystem attracts people here, and it doesn't make sense to decimate the few remaining herds indiscriminately.

Thank you for your time and consideration.

Patricia Grady

[redacted]

From: [redacted]
To: [redacted]
Subject: "Who really pays for wildlife in the U.S?"
Date: Monday, November 09, 2015 2:24:06 PM

- Attention:
- Mr. Jack Baylis, President
- Mr. Jim Kellogg, Vice President
- Mrs. Jacque Hostler-Carmesin, Member
- Mr. Anthony C. Williams, Member
- Mr. Eric Sklar, Member
- Sonke Mastrup

Most of you are doing a wonderful job, and we Californians are very grateful!
I am sending the following article for your information; I found it very enlightening. Since less than 6% of Americans hunt, we, the other 94%+ request (demand?) that OUR wildlife and Public Lands should no longer be managed for the benefit of a tiny minority. There are months when a non-hunter is taking their life and their pets lives into their hands by daring to use OUR Public lands! I think it may clarify your jobs when you realize exactly WHO you are managing OUR lands for.

http://www.mountainlion.org/featureimages/whopaysforwildlife/USA-O-NRWM-Smith-Molde-2014-Wildlife-Conservation-Management-Funding-in-the-US.pdf

"Who really pays for wildlife in the U.S?" Using public information about budgets of various conservation, wildlife advocacy, and land management agencies and non-profit organizations, published studies and educated assumptions regarding sources of Pittman-Robertson Act and Dingle-Johnson Act federal excise monies from the sale of sporting equipment, the authors contend that approximately ***95% of federal, 88% of non-profit, and 94% of total funding for wildlife conservation and management come from the non-hunting public.*** **The authors further contend that a proper understanding and accurate public perception of this funding question is a necessary next step in furthering the current debate as to whether and how much influence the general public should have at the wildlife policy-making level, particularly within state wildlife agencies...wildlife is unequivocally a public Wildlife Conservation... asset under the Public Trust Doctrine, a better understanding and definition of how wildlife management is financed in this country, particularly the portion attributable to the general public, would be of considerable help in deciding whether the general public's interest is adequately represented in our current wildlife management system.**

WILDLIFE CONSERVATION & MANAGEMENT FUNDING IN THE U.S. By Mark E. Smith1 Donald A. Molde2 October 2014 EXECUTIVE SUMMARY The authors present a novel approach to help answer the question "Who really pays for wildlife in the U.S?" Using public information about budgets of various conservation, wildlife advocacy, and land management agencies and non-profit organizations, published studies and educated assumptions regarding sources of Pittman-Robertson Act and Dingle-Johnson Act federal excise monies from the sale of sporting equipment, the authors contend that approximately 95% of federal, 88% of non-profit, and 94% of total funding for wildlife conservation and management come from the non-hunting public. The authors further contend that a proper understanding and accurate public perception of this funding question is a necessary next step in furthering the current debate as to whether and how much influence the general public should have at the wildlife policy-making level, particularly within state wildlife agencies. INTRODUCTION With increased awareness and interest of the general (non consumptive) public in controversial wildlife management issues such as fur trapping, predator control, trophy hunting, coyote killing contests and wolf reintroduction, a debate is before us as to whether the general public is or should be afforded a proper voice in wildlife management decisions. Sportsmen favor the current system, which places a heavy emphasis on their interests through favorable composition of wildlife commissions and a continued emphasis on ungulate management. Nonhuman predators (wolves, mountain lions, coyotes,

ravens and others) are disfavored by wildlife managers at all levels as competition for sportsmen and are treated as second-class citizens of the animal kingdom. Sportsmen suggest this bias is justified because “Sportsmen pay for wildlife,” a refrain heard repeatedly when these matters are discussed. Agency personnel and policy foster this belief as well. Do sportsmen really pay for wildlife? Is it a fact or an unfounded assertion or something in between? Are there ways of looking at financial and other information to test the merit of this claim? While wildlife is unequivocally a public Wildlife Conservation & Management Funding in the U.S. Smith & Molde, Oct. 2014 2 asset under the Public Trust Doctrine (see, for example, SCOUS 1842 and Horner 2000), a better understand and definition of how wildlife management is financed in this country, particularly the portion attributable to the general public, would be of considerable help in deciding whether the general public’s interest is adequately represented in our current wildlife management system. Summary of Findings While this question is not easy to answer and the information may be murky, we have devised a novel approach, using available public information and certain helpful assumptions to offer a perspective on this question, which, to our knowledge, has not been previously presented. The results are expressed both in terms of annual budgets by organization (Table 1) and acreages under management (Table 2). In summary, approximately 95% of federal, 88% of nonprofit, and 94% of total funding for wildlife conservation and management come from the non-hunting public. This runs counter to the common position promoted by many hunter-centric organizations and even to what state wildlife agencies often cite (e.g. Mayer, 2012). Another example of this is a motto of the Rocky Mountain Elko Foundation: “Hunting is Conservation.” Obviously hunting per se is not conservation, but they claim that hunting funds conservation, nearly exclusively. The data in Table 1 shows that the financial contribution from hunters is a small portion of the total. Of the 8 largest federally funded wildlife programs listed in the top half of Table 1, a total of $18.7 billion is spent annually on wildlife, land management and related programs (including hunter education). Approximately 5.3% of the combined operating budgets (top half of Table 1) and 4.9% of the land acquisition costs (Table 2) are funded by hunters or through hunting-related activities. The 10 largest non-profit conservation organizations contribute $2.5 billion annually to habitat and wildlife conservation; of this, 12.3% comes from hunters and 87.7% from the non-hunting public (bottom half of Table 1). Methodology In Tables 1 & 2 values have been assigned for the portion of funding derived from hunters or hunting-related activities. The difference between “hunter” and “hunting related” as well as the allocation of Pittman-Robertson and DingellJohnston Act funds are discussed in the section entitled Pittman-Robertson & Dingell-Johnson Acts. The allocations for the other items are discussed in the sections entitled General Tax Revenue and Duck Stamp Act. State funding was not considered in this study, in part because most state wildlife agency funding flows from the federal government (about 70% in Nevada’s case), and in part because it would be a task larger than our resources allowed. It is also generally true that the state funding (e.g. hunter license and tag Wildlife Conservation & Management Funding in the U.S. Smith & Molde, Oct. 2014 3 sales) is rarely adequate to cover the direct costs of administering the related programs; therefore, state-level funding can reasonably be classified as hunting or sportsmen services rather than wildlife management. State-owned public lands are considered in terms of the acreage under management in Table 2. Also not considered is the portion that each agency or organization actually spends on conservation versus other activities. For example, most state wildlife agencies spent only a small portion of their total funding on conservation. Other organizations, such as the government funded National Wildlife Refuge System and the donor funded Nature Conservancy, spend the great majority of their funding on conservation. This differential was ignored in our analysis. Table 1: Summary of Conservation Funding by Source (M = million US dollars) Activities Funded by Hunters Source Total Annual Funding, $ % of Total $ Activities Funded by Non-hunting Public, $ National Wildlife Refuge System (operating budget, see Table 2 for land purchase funding) $276M 4.6% $13M $263M Pittman-Robertson & Dingell-Johnson Acts Funds: Funding based on hunting activities Funding based on population $882M 14.5% 4.6% $128M $35M $719M USDA Wildlife Services $89M 4.6% $4 $85M USDI Fish & Wildlife $2,795M 4.6% $129M $2,666M US BLM $1,200M 4.6% $56M $1,144M US Forest Service $9,779M 4.6% $453M $9,326M National Park System $3,650M 4.6% $169M $3,481M SUBTOTAL Federal Funding $18,671M 5.3% $986M $17,685M 94.7% Nature Conservancy $859M 4.6% $40M $819M Land Trusts (all, except N.C) $535M 4.6% $25M $510M Wildlife Conservation Society $230M 4.6% $11M $219M World Wildlife Fund $204M 4.6% $9M $195M Ducks Unlimited $147M 99% $146M $1M The Conservation Fund $138M 4.6% $6M $132M Natural Resources Defense Council $123M 4.6% $6M $117M National Wildlife Federation $93M 4.6% $4M $89M National Audubon Society $89M 4.6% $4M $85M Rocky Mountain Elk Foundation $54M 99% $53M $1M SUBTOTAL Non-profit Organizations

$2,472M 12.3% $304M $2,168M 87.7% TOTAL Federal & Non-profits $21,143M 6.1% $1,290M $19,853M 93.9% In our analyses we included those agencies and organizations commonly considered by the public to have as their focus habitat and wildlife management Wildlife Conservation & Management Funding in the U.S. Smith & Molde, Oct. 2014 4 or conservation (e.g. U.S. Department of the Interior Fish & Wildlife Service (FWS), National Wildlife Refuge System, Nature Conservancy, Audubon). We also included agencies and organizations whose primary purpose is to conserve or manage the lands that host wildlife (the U.S. Forest Service (USFS), U.S. Bureau of Land Management (US BLM), the state equivalents, the various land trusts). The organizations that manage habitat, such as the US BLM and the many private land trusts, are included herein because wildlife requires habitat. The goals of these organizations include various combinations of habitat management and conservation, biological diversity (necessary to ensure robust populations), food and water supply, watershed protection, migration corridor management, and other issues critical to wildlife conservation and management. A potentially controversial choice was to include the U.S. Department of Agriculture Wildlife Services. Their core function is to control native carnivores such as wolves, bears, and coyotes, ostensibly to protect agriculture and improve hunter opportunity (Bruskotter, 2011). Arguably they accomplish neither since most livestock mortality is due to weather, birthing, and disease, while most wildlife mortality is due to lack of food, impacts to habitat, and disease. While predator control is wildlife management it is not conservation. Nevertheless, we have included their budget in our funding analyses. Table 2: Summary of Land Under Direct Management (M = million acres) Land Purchases Funded by Hunters Land Purchases Funded by Non-hunting Public Source Land Under Management, acres % acres acres National Wildlife Refuge System Funding based on hunting activities Funding based on population 150M 1.7% 4.6% 2.6M 6.8M 140.6M US BLM 248M 4.6% 9.9M 236.5M US Forest Service (note A) 193M 4.6% 7.7M 184.1M National Park Service 84M 4.6% 3.4M 80.1M State Lands (all states) 197M 4.6% 7.9M 187.9M SUBTOTAL State & Federal Funding 872M 4.9% 42.8M 829.2M 95.1% Nature Conservancy 119M 4.6% 4.8M 113.5M Land Trusts (all) 47M 4.6% 1.9M 44.8M SUBTOTAL Non-profit Organizations 166M 4.6% 7.7M 158.3M 95.4% TOTALS 1,038M 4.9% 50.5M 987.5M 95.1% Note A. The USFS indirectly or cooperatively manages 600M acres. Wildlife Conservation & Management Funding in the U.S. Smith & Molde, Oct. 2014 5 SOURCES OF FUNDING & ALLOCATIONS The sources of our funding and land acreage figures were, in most cases, the official websites for the named agencies or organizations. The total acreage under management by land trusts was obtained from the Land Trust Alliance (http://www.landtrustalliance.org). Pittman-Robertson Act and Dingell-Johnston Act revenue were obtained from the most recently published federal budgets for the Bureau of Alcohol, Tobacco, Firearms and Explosives (ATF) and US Fish and Wildlife Services (FWS), the agencies responsible for collecting and administering these funds, respectively. See the section entitled PittmanRobertson and Dingell-Johnston Acts for a detailed discussion of how those revenues were allocated, as well as a brief discussion of the two Acts. General Tax Revenue Most of the federal programs relevant to wildlife management and conservation are funded from general tax revenue such as personal and corporate income taxes. The key exceptions to this are the tax transfers made to the states under three well known acts (and their amendments): the Federal Aid in Wildlife Restoration Act of 1937 (more commonly known as the Pittman-Robertson Act or PRA), the Federal Aid in Sport Fish Restoration Act (Dingell-Johnston Act or DJA), and the Migratory Bird Hunting Stamp Act (Duck Stamp Act). Each of these acts is discussed in more detail in subsequent sections. Rather than attempt an allocation of general tax revenue funding to “hunter funding” and “non-hunting public funding” by some complex analysis of demographics, we chose the simpler, and possibly equally accurate, method of allocation based on the percent of the population who hunts. According to the US FWS (2013), there were 14,631,327 hunting licenses issued by all US states in 2013, down from 14,960,522 in 2012. There are two important bias in these statistics: hunters who purchased licenses in more than one state are counted for each state; and most states exempt youth from license requirements (e.g. Nevada hunters under the age of 12 years are not required to purchase a license, in some states the age is 16). We could not find any published analyses on either, so we have made no change to the data published by the FWS. It is likely that both figures are small and each acts to reduce the effect of the other. According to the US Census Bureau (2013), the US population in July 2013 was 316,128,839. Dividing that into the number of hunting licenses sold in 2013 suggests that 4.6% of the population, and therefore the same percentage of general tax revenue is paid by hunters. That figure has been used in Tables 1 & 2. An important side note is that while the US population increases annually, the number of hunting licenses sold is on the decline. Wildlife Conservation & Management Funding in the U.S. Smith & Molde, Oct. 2014 6 Duck Stamp Act Funding

of wildlife land gets a lot of attention among sportsmen and other outdoor enthusiasts. One of the most common to come up in discussion is the Duck Stamp program and the land that it has successful protected as refuges under the National Wildlife Refuge System. The federal government estimates that 1.9% of the 150,000,000 acres (or 2,850,000 acres) of land managed under this program was acquired with funds from programs including duck stamp sales (USFW, 2014; Lin, 2014). It has been estimated that collectors purchase 10% of duck stamps. Duck stamps allow free access to refuges that otherwise charge an entrance fee, and an unknown portion of the public purchases them for this purpose. To determine the total hunter-sourced portion of the National Wildlife Refuge System acreage, we combined 90% of the 1.9% with the hunter portion of general tax revenue (4.6%). Considering the four main federal agencies, the combined state-owned lands, and the collective non profits falling in the category of land trusts, there are 1.038 billion acres of wildlife habitat under conservation management, of which about 4.9% were funded by hunter and 95.1% funded by the non-hunting public. Pittman-Robertson & Dingell-Johnson Acts The process of determining the portion of the Pittman-Robertson Act (PRA) & Dingell-Johnson Act (DJA) excise taxes generated by hunting-related activities is both complex and imprecise. In the end, any such analysis can only be an estimate, since the revenue is not tracked in sufficient detail to allow a precise allocation. Our approach was to both recognize and minimize the biases created by our assumptions in these analyses. The approach taken in this study is briefly summarized here. Beginning in 1919, there has been an excise tax on firearms and ammunition (10 to 11% of the wholesale price). This tax was originally administered under the US Treasury, and the income went into the general fund. In 1937, the Pittman-Robertson Act transferred this tax to administration by the FWS for the exclusive purposes of wildlife management, hunting management, and hunter education. The Dingell-Johnston Act (1950), as amended by the Wallop and Breaux Act (1984), extended the excise tax to archery equipment, fishing supplies, recreational boat import duties, and marine fuel sales. PRA and DJA funding totaled $522 million and $360 million, respectively, for the 2013 fiscal year. Our analyses consider funding allocations in two portions: the first based on activity (hunting related versus non-hunting related), and the second on general population (hunters versus the non-hunting public). This section discusses the former; the latter uses the same allocation as for the other categories. We used this split approach for the PRA and DJA funds because firearms, ammunition, and archery equipment are purchased by both hunters and non-hunters and Wildlife Conservation & Management Funding in the U.S. Smith & Molde, Oct. 2014 7 these are used for both hunting and non-hunting purposes. Therefore, putting the total revenue into either the “hunter” or “non-hunting public” categories would have created a strong bias. The next step was to consider the nature of the purchases that generate the excise taxes collected. According to the ATF (Hogue, 2013), the PRA revenue is generated in the following proportions: o 31% from handgun (pistols and revolvers) production o 37% from long guns (rifles and shotguns) production o 31% from ammunition production o 1% from archery equipment production Dingell-Johnston Act revenue is generated in the following proportions, according to US DF&W statistics (Michigan): o 54% from motorboat fuel o 15% from small engine fuel o 16% from fishing equipment, tackle, trolling motors o 9% from interest on trust fund deposits o 6% from import duties on boats Of these funds, we next made an estimate of the portion of the revenue generated from hunting. We used a variety of sources of information to produce these estimates, principally the following. From ATF statistics on sales of firearms by type (ATF, 2011), we identified those types of firearms that are used principally for hunting. We used the following allocations: traditional rifles and shotgun sales were allocated to hunting; modern sporting rifles (including tactical shotguns) were allocated principally (but not exclusively) to non-hunting. The results was a combined allocation of 40% of long guns to hunting. Handgun sales were allocated 5% to hunting based on anecdotal evidence. Statistics for ammunition sales published by industry sources (LuckyGunner, 2012 & 2013; Bushmann, 2014) were used to determine the approximate percentage of sales by caliber. Each caliber was then assigned to one of three categories: principally hunting, principally non-hunting, or split. Examples of ammunition classified herein as principally for hunting include .243 Winchester, .30-06 Springfield and .308 Winchester (traditional deer and elk calibers). Principally non-hunting ammunition include .380 acp, 9 mm parabellum (aka 9mm Luger), .45 acp, .338 Lapua and .50 BMG. Calibers considered to be used for both hunting and non-hunting include shotgun shells in all gauges, .223 Remington, 5.56x45mm, 7.62x39mm, and large bore magnum handgun calibers such as .44 Remington Magnum and .500 S&W Magnum. Based on the relative sales statistics, considerably less than 20% of ammunition sales appear to be hunting related, but 20% was used in our analysis; this higher figure was used because 2013 represented an anomaly in sales statistics, with sales more Wildlife Conservation & Management Funding in the U.S. Smith & Molde,

Oct. 2014 8 heavily weighted towards self-defense and tactical than a multi-year average would suggest. Of the DJA revenue, the only category related to hunting is small engine fuel sales, some of which is likely used for waterfowl hunting. Our research did not find any data on allocation of these sales; we assumed 15% is derived from hunting-related activities. Table 3 summarizes the allocations and presents the estimated total funding generated by hunting-related activities. The estimate of 14.5% is consistent with the results published by other authors, a commonly cited range being 14% to 22% of the DJA funds alone (Lin, 2014), which equates to 8% to 13% of the combined PRA and DJA funds. Table 3: Allocation of Excise Tax Revenue Based on Activity (M = million US dollars) Proportion Derived from Hunting Excise Tax Source Activities Total Tax Revenue, $ % $ Handguns (revolvers, pistols) 164M 5 8M Long guns (rifles, shotguns, MSRs) 194M 40 78M Ammunition (all calibers) 163M 20 33M Archery equipment and supplies 4M 33 1M Fuel, motor boats 194M 0 0 Fuel, small engine 54M 15 8M Fishing equipment 59M 0 0 Interest on reserves 32M 0 0 Import duties on boats 22M 0 0 TOTALS $882M 14.5% $128M Another way to estimate the portion of PRA funds generated by hunting activity is to compare the number of guns used for hunting with total gun ownership. There are an estimated 270 to 310 million firearms in America (Krouse, 2012; GunPolicy; Crime Prevention Research Center, 2014). There are 14.6 million licensed hunters (FWS, 2013), though the number of licensed hunters who actually hunt is unknown. If we make an assumption that the average hunter owns 3 guns for hunting (e.g. two rifles and a shotgun), then 43.8 million guns are used for hunting, or 14.1 to 16.2% of the total. This would suggest that 8.8 to 10.1% of the combined PRA and DJA funds are huntingsourced. If we increase the per-hunter ownership assumption to 5 guns, the Wildlife Conservation & Management Funding in the U.S. Smith & Molde, Oct. 2014 9 portion of combined funding increase to 14.8 to 16.9%. Both ranges compare well with the 14.5% figure cited in Table 3. Possible Biases In Our Analyses of PRA & DJA Revenue A large portion of the PRA funds are set aside by law for hunter-specific uses rather than conservation or wildlife management. For example one half of the taxes generated from handguns and archery equipment are set aside exclusively for hunter education, which is principally about firearms safety (i.e., Hunter Ed); this totaled 15.9% of the PRA revenue in fiscal 2013. While one may reasonably argue that this money is not wildlife conservation funding, we have elected to ignore this issue. This creates a small bias in favor of the hunter-funding category. Fiscal 2013 was a record-setting year for firearm and ammunition sales, based on worries in the gun community about new federal gun control legislation following the Sandy Hook shootings and the reelection of President Obama. This increased the PRA funding, both in terms of the total dollars and the PRA percentage of the combined PRA and DJA revenues. This, in turn, increased the apparent hunting allocation over a multi-year average. At the same time the types of guns and ammunition which saw the greatest sales increases in 2013 were not traditional hunting equipment but rather modern sporting riflesa or MSRs), tactical shotguns, tactical and self defense ammunition. For example, 9mm handgun ammunition sales increased to 21.4% of the total in 2013 from 14.2% in 2012 (LuckyGunner, 2012 & 2013). According to the National Shooting Sports Foundation survey, “82% of recent purchases were AR-platform rifles” (NSSF, 2013). The disproportionate increase in sales of pistols, tactical long guns, and the related ammunition decreased the apparent hunting contribution. The net affect was not estimated. We applied 40% of long gun sales in 2013 to hunting sales, though the statistics suggest that this number should be lower. If, for example, the above NSSF quote is accurate and 82% of recent sales were AR platforms, and if AR platforms are principally not used for hunting as other surveys and anecdotal evidence suggest, the actual percentage allocated to hunting could be overstated by 10 percentage points or more. a Modern Sporting Rifles is an industry term for the class of rifles sometimes referred to assault rifles, assault weapons or tactical rifles. For the purposes of this paper the term is used for the class of rifles that include AR-10, AR-15, AK-47 and Uzi-style platforms and their variants. While many in the public and media seem to focus on the appearance, the most important features in terms of our use of this classification are the semi-automatic action, carbine length, and the use of a detachable magazine. Wildlife Conservation & Management Funding in the U.S. Smith & Molde, Oct. 2014 10 CLOSING COMMENTS Gill (1996) concluded that the narrowly based funding of state wildlife agencies has ‘‘blurred the essential distinction between public interest and special interest and inevitably eroded both scientific credibility and public trust.’’ We would argue that it is the perception not the reality that has blurred the distinction. For example, then director of the Nevada Department of Wildlife Ken Mayer wrote to the legislative sunset subcommittee “…the contribution to NDOW’s operating budgets from sportsmen is 79 percent of total funding” (Mayer, 2012). He was assuming that all of the federal excise tax transfers were hunter-sourced. This is a misrepresentation often used to manipulate public opinion

and influence policy. This narrative "…logically encourages those who pay via licenses and permits for the privilege of using wildlife to expect greater benefits…Because [it's believed that] hunters pay the bills, it is not surprising that they are given much attention and wield a great deal of influence…" (Jacobson et al, 2010). Modern wildlife management has wandered far from the original path of the Public Trust Doctrine and the North American Wildlife Conservation Model from which it flows (SCOUS, 1842; Horner, 2000). Smith (1980) identified three criteria that need to be met for the Public Trust Doctrine to be effective: 1. The general public must be aware of their legal standing with respect to public ownership of wildlife; 2. This standing and the rights associated with it must be enforceable against the government so that the public can hold it accountable; and, 3. Interpretation of these rights must be adaptable to contemporary concerns, such as biodiversity and species extinction. All three are impaired when the basis of public debate is a myth. It's time that we call for honest dialog from our state and federal agencies and transparency in wildlife policy making. ACKNOWLEDGEMENTS We would like to express our deepest gratitude to the many professionals who provided review, editing and critical input into the development of this paper. Especially noteworthy are Wendy Keefover of the Humane Society of the United States and Harley Shaw, retired, Arizona Game & Fish Department who provided invaluable review and editing support. REFERENCES Bruskotter, Jeremy T. (2011), "Rescuing wolves from politics: wildlife as a public trust resource." Science magazine, Vol. 333, pg. 1818-1829, September 30. 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Blessings, Jane Eagle

When people say, "I couldn't foster because it would be too hard to give a dog up." We say, "How can it be harder than knowing a dog died because no foster home stepped up?" and that's why we do it time and time again.