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Using eDNA to validate predation on native *Oncorhynchus mykiss* by invasive Sacramento pikeminnow *(Ptychocheilus grandis)*

Ken W. Jarrett*, Ethan Bell, Emily A. Wilson, Tom Dudley, and Carolyn M. Geraghty

Stillwater Sciences, 895 Napa Ave, Suite B-4, Morro Bay, California, 93442, USA (KWJ, EB)

Marine Science Institute, University of California, Santa Barbara, CA 93106, USA (EAW, TD)

Morro Bay National Estuary Program, 601 Embarcadero, Suite 11, Morro Bay, California, 93442, USA (CMG)

**Correspondent: ken@stillwatersci.com*

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Traditional methods for assessing fish predation have the potential to underestimate the occurrence of important prey items due to rapid digestion and evacuation rates (Deagle et al. 2005, Ley et al. 2013). Visual examination of gut contents is a common method used to determine fish diet and predation rates of fish (Hyslop 1980, Hartleb and Moring 1995), but in many cases the results only represent a short window of feeding activity. For example, laboratory studies have reported larval and early life stage fish become unidentifiable after less than two hours post-ingestion at water temperatures of 16–20°C (Schooley et al. 2008, Legler et al. 2010).

Initial investigations have demonstrated that genetic analysis can lengthen the gut content detection window for prey items (Ley et al. 2013). Studies analyzing DNA collected from stomach samples have been able to detect and identify larval fish species up to 48 hours after ingestion even when the gut appeared empty (Hunter et al 2012), while juvenile fish DNA has been detected more than 100 hours after ingestion (Brandl et al. 2016). Prey DNA detection half-life (where prey DNA is detected in half of the predators after ingestion) occurred at 26 hours for larval Delta smelt (*Hypomesus transpacificus*) fed to Mississippi silverside (*Menidia audens*) and 66 hours for whole juvenile Chinook salmon (*Oncorhynchus tshawytscha*) fed to striped bass (*Morone saxatilis*), under controlled temperatures (18°C) using qPCR DNA analysis (Brandl et al. 2016).

In this study, we assessed evidence for predation by non-native Sacramento pikeminnow (*Ptychocheilus grandis*) (hereafter referred to as "pikeminnow") on California red-legged frog (CRLF) (*Rana draytonii*) and steelhead/rainbow trout (*Oncorhynchus mykiss*) in Chorro Creek using traditional assessments and genetic analysis of samples collected from pikeminnow stomachs.

Chorro Creek is a tributary to Morro Bay on the California central coast (Figure 1). The watershed drains 111 km^2 and provides important habitat for two federally listed aquatic species, CRLF and *O. mykiss*. The Chorro Creek watershed has several factors that provide a higher potential for *O. mykiss* recovery and resiliency than in other nearby watersheds, including perennial and continuous flows in the mainstem downstream of a waste water treatment plant that provides year-round migratory connectivity to a productive estuary (Morro Bay), dense riparian canopy, moderate summer water temperatures, and a relatively small urban footprint. However, Chorro Creek also supports a self-sustaining population of non-native pikeminnow, which have been reported to prey on juvenile *O. mykiss* and frogs (Brown and Brasher 1995, Brown and Moyle 1996, Nakamoto and Harvey 2003). The presence of pikeminnow in the Chorro Creek watershed may inhibit *O. mykiss* recovery by reducing juvenile survival through predation and competition for food and habitat. Although other non-native fish species have been observed in Chorro Creek (e.g., largemouth bass [*Micropterus salmoides*]), they have not established stable populations and are only rarely observed (D. Michniuk, CDFW, pers. comm. 2017). There are no native predators to *O. mykiss* in Chorro Creek.

Pikeminnow larger than 200 mm (Standard Length, [SL]) feed almost exclusively on fish and crayfish (Brown and Brasher 1995). Pikeminnow typically reach this size by the end of their third year (Moyle 2002). Moreover, as juveniles, pikeminnow have a diet and habitat distribution similar to juvenile *O. mykiss*, leading to likely competition for food

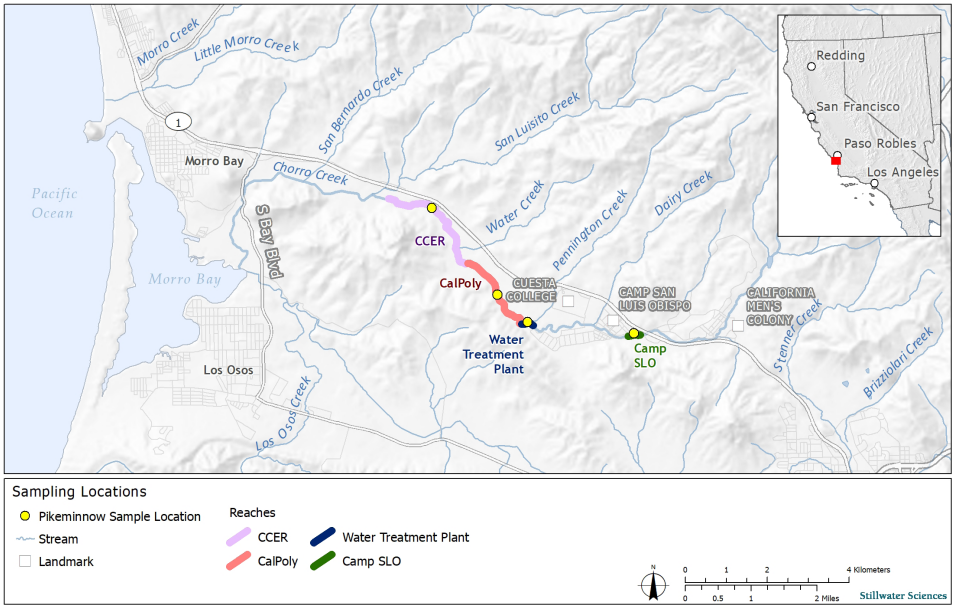

FIGURE 1.—Chorro Creek watershed and Sacramento pikeminnow sample locations.

between juveniles of these two species. Reese and Harvey (2002) found a reduction in *O. mykiss* growth of more than 50% when pikeminnow were present compared to growth without pikeminnow.

The ability to identify fish prey items using visual examination of pikeminnow stomachs is expected to be limited by their high digestive rates. Pikeminnow have rapid digestion rates with gastric evacuation times reported to range from 36 hours at 10°C, 17 hours at 15°C, and 14 hours at 20°C after consuming juvenile Chinook salmon (*Oncorhynchus tshawytscha*) (Vondrcek 1987). This suggests that in Chorro Creek, where water temperatures range from approximately 10°C during the winter to 20°C during the summer (Kitajima 2016), a juvenile *O. mykiss* consumed by a pikeminnow would have exited the stomach after about 17 hours and the ability to visually detect a fish prey item and identify it to species would be significantly shorter.

Pikeminnow were captured from pool habitats within four sections of Chorro Creek in fall 2017 and spring 2018 (Figure 1 and Table 1). Sampling efforts during fall were conducted to coincide with low flows in Chorro Creek, while spring efforts were conducted to coincide with *O. mykiss* fry emergence (based on visual observations from snorkel dives). Piscivorous-size (> 200 mm SL) pikeminnow were targeted by angling with lures that imitate juvenile *O. mykiss* or were captured using spearfishing. Only pikeminnow greater or equal to 165 mm SL were retained for analysis. After capture, the location, species, fish length (SL), and sample ID were recorded. All captured pikeminnow were euthanized (following AVMA [2013] guidelines), measured, and processed. To conduct stomach analysis and collect DNA samples, biologists used a pair of sterile gloves before processing fish, and gloves were disposed of after handling each fish. A sterile scalpel was used for the initial cut into the body cavity, after which the scalpel was disposed of. Once the cavity was open, a sterile scalpel was used to remove and then open the stomach. All recognizable prey items were recorded. Large items were removed, and the stomach and intestines were rinsed with 95% ethanol. Runoff from the stomach and intestine rinsing was captured in a 5 mL Eppendorf tube and stored on ice for DNA analysis (described below). Each fish was processed with new sterile items (i.e., gloves and scalpels). A single stomach sample was also obtained from a Sacramento sucker (*Catostomus occidentalis*) incidentally captured during fall 2017. This fish was used as a control sample during analysis because Sacramento sucker are not piscivorous, and therefore would not be expected to contain DNA from *O. mykiss* or CRLF.

Vials containing the stomach contents were wiped down with 10% bleach and 70% ethanol twice to remove DNA from the outside of the container. The stomach content samples were thoroughly mixed, and 2 mL of the sample was poured into a new 5 mL vial for DNA extraction. One stomach sample included a fish which could not be visually identified to species; therefore, two DNA extraction samples were collected from this stomach: a tissue sample of the unknown fish and the ethanol rinse runoff from the stomach. Samples were placed under a laminar flow hood to evaporate all ethanol from the samples. Three negative extract controls were created using fresh 100% ethanol and followed the same extraction procedure with the field samples to evaluate any potential cross-contamination between samples. Once the ethanol was evaporated, DNA was extracted from the samples using Qiagen's DNeasy Blood and Tissue Kit, modifying the manufacturer's protocol by using a larger volume of the initial buffer and lysing solutions to account for the larger volume of starting material. Each DNA extract was eluted into 200 µL of AE buffer solution and stored at -20°C until further analysis.

Table 1.—Visual and DNA detections of prey items found in stomach samples collected from Sacramento pikeminnow in Chorro Creek, California during fall 2017 and spring 2018.

Sample date	Capture location[a]	Species	Standard length (mm)	Visual assessment of gut contents[b]	*O. mykiss* DNA copies detected[c]
Fall 2017 sampling effort					
9/9/17	CCER	Pikeminnow	385	crayfish	No
9/9/17	CCER	Pikeminnow	335	empty	Yes (10.2)
9/9/17	CCER	Pikeminnow	345	crayfish	No
9/9/17	CCER	Pikeminnow	280	crayfish	No
9/9/17	CCER	Pikeminnow	238	crayfish	No
9/9/17	CCER	Pikeminnow	340	empty	No
9/9/17	CCER	Pikeminnow	370	empty	No
9/9/17	CCER	Pikeminnow	325	empty	Yes (27.1)
9/9/17	CCER	Pikeminnow	220	empty	No
9/13/17	CalPoly	Pikeminnow	390	empty	No
9/13/17	CalPoly	Pikeminnow	285	crayfish	Yes (3.8)
9/13/17	CalPoly	Pikeminnow	260	empty	No
9/13/17	CalPoly	Pikeminnow	287	crayfish	No
9/13/17	CalPoly	Pikeminnow	285	crayfish	No
9/14/17	CalPoly	Sacramento sucker	195	empty	No
Spring 2018 sampling effort					
4/20/18	CalPoly	Pikeminnow	220	empty	No
4/20/18	CalPoly	Pikeminnow	355	empty	Yes (9.0)
4/20/18	CalPoly	Pikeminnow	200	empty	No
5/4/18	CalPoly	Pikeminnow	290	empty	No
5/4/18	CalPoly	Pikeminnow	280	crayfish & beetle	No
5/4/18	CalPoly	Pikeminnow	270	crayfish	No
5/4/18	CalPoly	Pikeminnow	360	multiple crayfish	No
5/8/18	CalPoly	Pikeminnow	325	crayfish	No
5/9/18	Camp SLO	Pikeminnow	250	crayfish parts	Yes (6.3)
5/9/18	Camp SLO	Pikeminnow	280	whole crayfish	Yes (3.5)
5/9/18	Camp SLO	Pikeminnow	300	crayfish parts	No
5/9/18	Camp SLO	Pikeminnow	360	crayfish	No
5/9/18	Camp SLO	Pikeminnow	300	crayfish	No
5/9/18	Camp SLO	Pikeminnow	320	crayfish	No
5/9/18	Camp SLO	Pikeminnow	290	empty	No
5/9/18	Camp SLO	Pikeminnow	280	crayfish	No
5/9/18	Camp SLO	Pikeminnow	280	empty	No

Table 1 continued.

Sample date	Capture location[a]	Species	Standard length (mm)	Visual assessment of gut contents[b]	*O. mykiss* DNA copies detected[c]
5/9/18	Camp SLO	Pikeminnow	248	empty	No
5/10/18	Water Treatment	Pikeminnow	305	crayfish	No
5/10/18	Water Treatment	Pikeminnow	315	unidentified fish	Yes (85,350)[d]
5/10/18	Water Treatment	Pikeminnow	322	crayfish	No
5/10/18	Water Treatment	Pikeminnow	270	empty	No
5/10/18	Water Treatment	Pikeminnow	290	empty	No
5/10/18	Water Treatment	Pikeminnow	220	empty	No
5/10/18	Water Treatment	Pikeminnow	165	empty	No
5/10/18	Water Treatment	Test Blank	na	na	No

[a] Locations shown on Figure 1.
[b] Visual assessment of gut contents includes items identified visually from dissected fish stomachs.
[c] Number of DNA copies detected is included in parenthesis for positive *O. mykiss* detections.
[d] Value reported is from stomach sample, a tissue sample from the unidentified fish was also analyzed and was positive for *O. mykiss* DNA with 495,500 DNA copies detected.

Species-specific DNA markers were used to assess the presence of *O. mykiss* (Brandl et al. 2015) and CRLF (Halstead et al. 2018) in the stomach samples. Markers were developed from mitochondrial genes cytochrome oxidase 1 and cytochrome b for *O. mykiss* and cytochrome b for CRLF. Brandl et al. (2015) and Halstead et al. (2018) demonstrated that their marker sets were specific to the targeted species and do not amplify DNA from closely related species that may occur within the study area. All assays used TaqMan MGB probes (Life Technologies) in singleplex reactions on a StepOnePlus quantitative PCR machine (Applied Biosystems). To ensure maximum detection, we optimized primer and probe concentrations for our assay protocol using a synthetic double-stranded DNA (gBlock) (Integrated DNA Technology, San Diego), created from the targeted species' DNA sequences available from the NCBI database. The primer and probe concentrations for each species-specific marker was optimized for use in a 25 µL reaction using the qPCR thermal cycles 50°C for 2 minutes, 95°C for 10 minutes, and 50 cycles of 95°C for 15 seconds and 60°C for 1 minute, as follows:

O. mykiss optimized reaction: 5µL of template DNA, 0.6 µM forward primer, 0.3 µM reverse primer, 0.2 µM probe, 1x Taqman Environmental Master Mix 2.0.

CRLF optimized reaction: 5µL of template DNA, 0.3 µM forward primer, 0.6 µM reverse primer, 0.25 µM probe, 1x Taqman Environmental Master Mix 2.0.

Each marker was tested on tissue from the targeted species to confirm positive detec-

tion. Trials were performed to test the sensitivity of each marker using the gBlock with the optimized primer and probe concentrations. Serial 1:5 dilutions were tested in duplicate using reactions with a high concentration, that ranged from over 20 million DNA copies (5E-03 ng/reaction) for both species down to 27 thousand DNA copies (5E-06 ng/reaction) for CRLF and 35 thousand DNA copies (5E-06 ng/reaction) for *O. mykiss*, and a low concentration, that ranged from approximately 30 copies (5E-09 ng/reaction) to less than one DNA copy per reaction (5E-11 ng/reaction), for both species.

Samples were tested for PCR inhibitors using an internal positive control assay (TaqMan Exogenous Internal Positive Control, Applied Biosystems) in their initial assay. All stomach content samples were tested in duplicate for *O. mykiss* and CRLF. Each assay plate included the set of field samples, three extract negative controls, three negative PCR template controls, and three 1:10 dilution standards run in triplicate from the synthetic DNA fragments. Dilution standards were based on previously described sensitivity trials and included the lowest concentrations that were consistently detected for each marker Samples were prepared in a clean room and moved into the post-PCR area for loading of the standards. Separate laboratory equipment was dedicated to either the pre- or post-PCR stations, which were in separate rooms to avoid potential introduction of high copy number material (synthetic gBlock DNA and amplicon) into field or controls samples. A sample was considered positive if either replicate displayed amplification before 45 cycles. Fifty cycles were included in the PCR cycle protocol to visually confirm that any positives after 40 cycles developed a complete amplification curve.

Stomach samples were analyzed from 39 pikeminnow captured in Chorro Creek, including 14 fish captured in fall 2017, and 25 fish captured in spring 2018. Captured pikeminnow ranged in size from 165 to 390 mm (SL). Based on growth rates reported in Moyle 2002, the size range of these fish correlates to fish ranging from two-years to over five-years in age. Visual observations of pikeminnow stomach contents generally identified stomach contents as either empty or containing crayfish. Only one pikeminnow was observed to have a fish in its stomach but the fish could not be visually identified to species due to the level of digestion.

Trials to test sensitivity showed positive detections during each of the high concentration dilution series replicates for both CRLF and *O. mykiss*. The low DNA concentration dilutions detected CRLF with only one DNA copy present in the reaction, whereas *O. mykiss* presence was detected with only four DNA copies present (Table 2). The positive control tissue samples from *O. mykiss* and CRLF amplified using their respective marker. The standard curves for all runs had efficiencies between 80-102%, $R^2 \geq 0.98$, and intercepts between 38 and 41 cycles.

Oncorhynchus mykiss DNA was detected in seven of the thirty-nine pikeminnow stomach samples (18%) (Table 1), confirming pikeminnow predation of *O. mykiss* in Chorro Creek. Crayfish parts were visually observed in 20 of the pikeminnow stomach samples (51%). The stomach sample from the pikeminnow with the fish in its stomach that could not be visually identified had the highest number of *O. mykiss* DNA copies detected while the tissue sample from this fish amplified nearly 500,000 DNA copies (Table 1). The proportion of pikeminnow stomach samples with positive detections for *O. mykiss* was similar in spring (21%) and fall (16%) (Table 3). No samples were inhibited, and all positive samples amplified before 38 cycles. No extract controls or negative template PCR controls were positive for *O. mykiss* DNA. No stomach contents were positive for CRLF.

Table 2.—Summary of the detection trials for low concentrations of the targeted synthetic DNA sequences (gBlock) for CRLF and *O. mykiss*. Values represent the number of DNA copies or ng of DNA per qPCR reaction. Each DNA concentration was tested in duplicate and any detection is listed with its associated Ct value(s).

Detection Trial	DNA Concentration	Detection[a]
CRLF		
#1	27 DNA copies (5E-09 ng)	Yes (Ct: 34.0; 33.2)
#2	14 DNA copies (2.5E-09 ng)	Yes (Ct: 33.7; 32.8)
#3	3 DNA copies (5E-10 ng)	Yes (Ct: 35.5; 35.0)
#4	1 DNA copy (2.5E-10 ng)	Yes (Ct: 36.8; 34.8)
#5	<1 DNA copy (5E-11 ng)	Yes (Ct: 36.7)
O. mykiss		
#1	35 DNA copies (5E-09 ng)	Yes (Ct: 33.7; 35.1)
#2	18 DNA copies (2.5E-09 ng)	Yes (Ct: 34.7; 36.0)
#3	4 DNA copies (5E-10 ng)	Yes (Ct: 37.0)
#4	2 DNA copies (2.5E-10 ng)	No
#5	<1 DNA copy (5E-11 ng)	No

[a] Positive detections are listed with the associated Ct value for each positive detection out of two replicates

In this study, visual gut observations revealed no *O. mykiss* in pikeminnow stomachs, but DNA detections found *O. mykiss* DNA in 7 of 39 pikeminnow stomach samples. Visual assessments found crayfish in twenty pikeminnow gut samples (51%), but crayfish exoskeletons and statoliths are more resistant to digestion than fish parts, requiring nearly twice as long to digest compared to fish (Schneider 1973). This suggests that studies using only a visual examination of the gut contents may bias the extent of predation on specific prey items based on the type of prey item being assessed and the time of sampling.

Results of this study suggest that genetic analysis of fish diet items is more reliable than the use of visual analysis. The methods of detection used in this study, allowed us to detect individual species from a slurry of mixed prey items found in stomach samples with eDNA while, historically, DNA analysis of stomach content required pieces of tissue from prey items and each piece had to be tested individually to identify the specific prey item. While visual observation may only provide reliable detection of prey items within a few hours of consumption (Schooley et al. 2008, Legler et al. 2010), qPCR can detect prey items consumed for up to a few days (Hunter et al 2012, Brandl et al. 2016). The ability to significantly extend the detection period of specific prey items makes qPCR a valuable technique for assessing fish diet and predation.

Researchers are still grappling with the potential for false positive and false negative eDNA results. Several recent papers have modeled the likelihood of false positives and negatives using eDNA (Lahoz-Monfort et al. 2015, Guillera-Arroita et al. 2017, Dorazio and Erickson 2017). Each of these papers explored a different approach and mathematical model, depending on the experimental design (e.g., whether they had known positive and

Table 3.—Proportion of Sacramento pikeminnow stomach samples with positive detections of O. mykiss DNA during fall 2017 and spring 2018 collection efforts in Chorro Creek, California.

Effort	Pikeminnow stomach samples	*O. mykiss* Detections	Proportion of samples with positive detections
Fall 2017	14	3	21%
Spring 2018	25	4	16%
Total	39	7	18%

negative field samples). A widely accepted model to accurately estimate the likelihood of false positives and false negatives has not yet been established.

In this study, the potential for false positives was controlled for by using sterile single use equipment at each step of eDNA sample collection and negative sample controls were used at each step of the eDNA process. None of the negative controls (i.e., stomach content DNA extraction or stomach content PCR template controls) were positive for any of the qPCR assays. This indicates that there was no contamination in the samples and provides confidence in the positive samples. The potential for false negatives were reduced by using highly sensitive markers and by processing samples in duplicate. Since DNA in stomach samples is expected to be much more concentrated than DNA in water samples, duplicate processing seemed sufficient to detect our target specie. However, since some of the samples had a positive detection for only one out of the two replicates, there is potential that additional samples may have had a positive detection if a third round of replication had been performed.

Pikeminnow predation rates appeared similar between seasons, although the overall sample size was low. Predation rates are likely to vary throughout the year in response to seasonal shifts in water temperature, metabolic rates, and variation in juvenile *O. mykiss* abundance associated with periods of outmigration and fry emergence. DNA detections do not indicate the size or number of prey items consumed. Therefore, positive DNA detections may have resulted from consumption of one *O. mykiss* or multiple *O. mykiss* per pikeminnow. Based on the high predation observed in this study, the *O. mykiss* population size in the watershed is likely being limited as a direct result of pikeminnow predation. Furthermore, individuals that remain are likely those that have found refuge in available cover or reside in habitat less optimal for pikeminnow, such as shallow runs and riffles.

There is a remote potential for *O. mykiss* DNA to end up in crayfish which may scavenge on dead *O. mykiss*. However, the likelihood of this occurring is expected to be extremely low because it requires a crayfish to find and consume a dead *O. mykiss*, for that crayfish to be consumed by a pikeminnow, and for the *O. mykiss* DNA to remain intact after being in two digestive tracts. The likelihood of this occurring once, let alone on seven separate occasions, is unlikely. Crayfish parts were observed in seventeen pikeminnow stomach samples where *O. mykiss* DNA was not detected, and crayfish were observed in three of the seven samples that had positive detections for *O. mykiss* DNA.

Our results did not detect any CRLF from the pikeminnow samples. Frogs are reported to be an important prey item for pikeminnow in the Eel River (Brown and Moyle 1996). During a prior study, a frog was found in the stomach of a pikeminnow within Chorro Creek, although, it could not be visually identified to species (Stillwater Sciences, unpublished data). CRLF tadpoles and egg-masses are most susceptible to predation and were

likely present at the locations sampled during the spring but not during the fall. Tadpoles and eggs are expected to digest at a higher rate than *O. mykiss* because of their soft bodies, which may make it more difficult to detect these prey items through DNA techniques and nearly impossible through traditional visual techniques. Additionally, CRLF may be less abundant than *O. mykiss* in this system based on fish monitoring efforts (Stillwater Sciences 2017 and 2018). It is possible that we may have detected CRLF in pikeminnow stomachs if we had a larger sample size.

Further research to determine DNA detection half-life specific to *O. mykiss* consumed by pikeminnow would help estimate predation rates for these predator-prey interactions. Our method was successful for use on non-listed species which can be sacrificed, but a modified approach may be required when sacrificing the predators is not feasible or desirable. For example, others have applied similar techniques to samples collected using nonlethal methods such as gastric lavage on species of concern (Barnett et al. 2010) to detect prey components.

The use of DNA samples can be used to assess predation of *O. mykiss* by pikeminnow and may be more reliable than visual examination of gut contents. In this study, pikeminnow predation levels on *O. mykiss* are likely substantial based on the proportion of positive DNA samples while the visual inspections suggested otherwise.

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