

COP Format for Project Final Report

A. Grant Number: 102263

B. Amount of Grant: \$42,981

C. Project Title: Assessment of Genetic Diversity and Connectivity in Fish Replenishment Areas in the Hawaiian Yellow Tang (*Zebrasoma flavescens*).

D. Abstract: The Hawaiian Yellow tang, *Zebrasoma flavescens* comprises 76% of fish collected on the island of Hawai'i for the aquarium trade. In 1999 the density of Yellow tangs was roughly 43% lower in areas where collection was allowed versus areas where collection was prohibited. Ongoing research so far indicates that recruitment of tangs, into Fish Replenishment Areas (FRAs) established along the west coast of Hawai'i in 2000, is beginning to increase in the absence of collection pressures.

Many coral reef fish exhibit a pelagic (oceanic) larval stage that can potentially interconnect geographically distant populations by oceanic currents. As such, it is possible that just a few major breeding areas (sources) are responsible for eventual recruitment of Yellow tangs into many different coastal ecosystems on a random basis. However, localized currents and larval behaviour have been proposed as possible mechanisms that may restrict the extent of mixing between sub-populations of fish. In this case, different source areas influenced by different ocean currents, such as the eddy system that forms around the island of Hawai'i, may foster genetically different sub-populations: An important consideration when developing management strategies.

We propose to undertake a genetic assessment of the Yellow tang from Hawai'i in order to further our understanding of recruitment and to identify sources of genetic diversity (if any) among Hawaiian Yellow tangs. This research would be carried out by the comparison of mitochondrial *Cytochrome b* gene and D-loop sequences as well as nuclear DNA RAPDs in adult fish and recruits collected from the west coast of Hawai'i. Additionally, fish from Hawaii's east coast and locations on another island should be included as a part of this study and would provide further information about population structure and recruitment from a statewide perspective.

This research would provide critical additional information required for management and planning of FRAs on the west coast of Hawai'i by the identification of genetically diverse and/ or genetically important sub-populations of Yellow tang. It would also provide new and advanced tools for fisheries monitoring and development of future FRAs throughout Hawai'i. Further, it would provide a basis for continuing genetic population research in tangs and serve as a model for other important reef organisms.

E. Executive Summary

I. Work Accomplishments: (as related to project objectives and schedule for completion)

a. In accordance with the goals and objectives outlined in the revised proposal, we have completed;

1. Construction of a microsatellite DNA library for the Yellow tang. This library consists of four bacterial clone libraries, each enriched for a different tetranucleotide microsatellite motif. The library is housed at Washington State University in a -80°C freezer. Construction of the library also entailed the design and optimisation of polymerase chain (PCR) primers for approximately 35 polymorphic microsatellite sequences, which were identified during the library construction process.

2. Sample collection and DNA extraction from approximately 400 Yellow tangs collected from Fish Replenishment Areas along the west coast of Hawai'i. Several samples were collected from the Maui Ocean Center, and DNA extracted from these also. An additional 120 adult samples were collected, and DNA extracted, representing the main juvenile collection sites.

3. Purification and quantitation of all DNA samples. The DNA has been transferred into 96 well microplate format and is stored at -20°C until required for analysis.

4. Fluorescent PCR primers have been developed and are optimised for analysis of microsatellite loci in the Yellow tang. Additionally to our proposal, we successfully tested the Yellow tang PCR primers in other coral reef species. This finding is of great interest to the Hawaiian scientific community and should form the basis of a subsequent funding proposal.

5. Microsatellite DNA electrophoresis and generation of raw data for 400 juvenile and 120 adult Yellow tang samples is complete. The final stage of this project will focus on population analysis of the microsatellite data and is expected to be completed by mid December.

6. We have surpassed original project objectives. In addition to analysis of all juvenile and adult samples as originally planned we have doubled sample numbers and included other species in this analysis.

7. Microsatellite DNA electrophoresis of adult tang samples has taken longer than anticipated but is complete as of March, 2005.

8. Manuscript preparation is under way – currently there are 2 manuscripts in preparation with a third pending continued statistical analysis.

9. Continuing work includes the following;

a. Computer-aided population statistical analysis is currently being undertaken.

2. Applications:

a. Presentations have been prepared and submitted to each of the quarterly HCRI meetings to date. The final presentation was prepared by Noakes and presented by Walsh in Honolulu. A short information session was also presented to a college class by Noakes during this period. A manuscript is in preparation for publication submission, explaining the characterisation of the microsatellite library and detailing variability at microsatellite loci in the Yellow tang. A short communication is also in preparation, detailing the usefulness of Yellow Tang microsatellite PCR primers in other coral reef fish species.

b. Interest generated during Noakes' visit to Hawai'i is has provided fruitful collaborative links between WSU Vancouver and UH. The research findings, including the microsatellite library resource, generated by this project are being utilised in continuing work in similar fish species in Hawai'i. The additional research findings in other coral reef species will benefit the Hawaiian research community and some preliminary research is presently being undertaken in Hawai'i that is based on the research findings of our project thus far. A funding proposal has been prepared by Jeff Eble *et al* (2005) that will use the microsatellite library and PCR primers generated and tested in this project.

c. The following data has been generated but not yet published;

1. DNA sequences of approximately 100 microsatellite clones from the library.
2. Approximately 270 oligonucleotide PCR primer sequences have been developed for Yellow tang microsatellites. Of these, 34 have been synthesised for use in fluorescent PCR analysis.
3. Eight Yellow tang individuals have been tested for each of the 34 synthesised primers and examined for polymorphism (see attached example agarose gel electrophoresis).
4. Nine of the above PCR primers have been selected and are presently being optimised for fluorescent PCR analysis in 400 juvenile and 120 adult Yellow tang samples. This data has been submitted to NOAA for archive.
5. Three PowerPoint presentations have been prepared, presented, and copies submitted to HCRI.

3. Expenditures:

a. There are no outstanding expenses. All continuing work will be undertaken with no addition expense.

4. Purpose

A. Relocation of Noakes to Australia created a 6 months delay in the final stage of the project. We are continuing work on the project without further expense.

B. This research aims to improve our understanding of the population structure and recruitment of the Hawaiian Yellow tang as directed by HCRI-RP priority #5. It strives to provide valuable information on the genetic composition of *Z. flavescens* and population subdivision, if any, on the west coast of Hawai'i, as well as intra-island and between islands. Additionally, by examining adults as well as recruits we expect to be able to comment on the genetic relationships between the two, and hence, connectivity among populations. Such research would provide additional and critical information required to assist in the future design and implementation of FRAs through the identification of genetically similar and dissimilar populations. In pursuit of this goal we propose to sequence the mitochondrial *Cytochrome b* gene and D-loop, and to generate fingerprint patterns using the randomly amplified polymorphic DNA (RAPD) technique. Our specific objectives are:

1. **Examine genetic diversity in mt DNA.** Samples of adult and newly recruited Yellow tangs collected from several FRAs along the west coast of Hawai'i, and from Hilo Bay and O'ahu, will be surveyed for genetic variation using DNA sequence analysis. The mitochondrial *Cytochrome b* gene and D-loop are the most rapidly evolving regions of the mitochondrial genome. As such they are useful for measuring genetic variation within a species. These regions will be sequenced and the genetic relationships among the different populations will be estimated from this data. Genetic relationships between resident adults and recruits will also be established.
2. **Examine genetic diversity in nuclear DNA.** The Yellow tang samples will be assessed for genetic variations in nuclear DNA using the RAPD technique. Nuclear DNA contains larger non-coding (neutral) regions than mt DNA. Analysis of neutral DNA in addition to mt DNA will yield a more comprehensive and accurate measure of genetic variation. The DNA will be amplified using a set of 10 random primers and genetic relationships among the different populations will be estimated from this data for comparison with the mitochondrial sequence data.
3. **Integration of genetic data with FRA monitoring for MPA management.** The genetic data provided by this research will provide critical additional input to FRA monitoring conducted by the WHAP. Specifically, information about genetic similarity and relatedness within and among geographic regions and FRAs, and the relationships between resident adults and recruits within and between each site, will contribute to our knowledge of the recruitment process. Also, such information can be used to support the design and development of future MPAs as well as contribute to the ongoing monitoring of FRAs provided by the WHAP. The identification of unique sources of genetic diversity among geographic locations represents a most valuable contribution to the management of Hawaii's marine resources.

5. Approach

A. Evaluating connectivity between sample sites in west Hawai'i, and at other sites, by examining genetic variation in the Yellow tang will provide advanced tools and crucial support for future MPA design. We propose to gather baseline genetic information for Yellow tangs in Hawai'i in an attempt to describe the genetic variation and biogeography of the species. This work would be carried out in collaboration with the West Hawai'i Aquarium Project (WHAP). In pursuit of our goal and objectives we will use standard molecular genetic techniques and analyses that have been successfully applied in many other fish species, including several other acanthurids. These include DNA extraction from fin tissue, the polymerase chain reaction (PCR), automated DNA sequencing and sequence analysis. Our approach will entail sequencing the D-loop and *Cytochrome b* gene in mt DNA as well as generation and analysis of randomly amplified polymorphic DNA (RAPD). These techniques will be conducted using both adult and newly recruited fish in order to obtain information for assessment of connectivity between populations. The mitochondrial region encompassing the D-loop and *Cytochrome b* gene have been consistently demonstrated to be one of the most rapidly evolving areas of the genome and are particularly useful for genetic population studies. The RAPD technique will allow evaluation of neutral DNA and will complement the mt DNA studies. Both these techniques have been utilised most especially in organisms for which little or no genetic information is available, such as the Yellow tang.

The following specific methods will be used to address the above objectives:

1: Sample collection.

This study will utilise samples collected as part of another study conducted by Walsh and Tissot as a part of the WHAP. Between 30 and 50 each of adult and newly recruited fish will be utilised from each sample site. Fish are collected by netting, are shortly thereafter euthanased by immersion in an ice-water slurry, and transported back to the lab. For DNA extraction, small (10 mm x 10 mm) pieces of the caudal and dorsal fins will be taken from frozen fish and placed into ethanol for transport from Hawai'i to WSU Vancouver. Approximately half of the tissue from each sample shall be processed by digestion at 37°C with DNA extraction solution. The remaining fin ray material is discarded and DNA is precipitated from the remaining solution with the addition of ethanol. The DNA is rinsed with 70% ethanol and the dried DNA is resuspended in ultra-pure water. Spectrophotometric measurement will be used to calculate DNA concentration and purity for all samples. All purified DNA samples and remaining fin material will be stored at 4°C. Sample information, including field sample number, collection site, collection date, size of specimen, specimen collector, laboratory sample number, DNA extraction date, DNA concentration and DNA purity will be entered into a Microsoft Access database for archive and retrieval by resource managers and for future reference and use in subsequent studies.

2: Microsatellite library construction.

Colony production

The bacterial cultures included in the accompanying package were produced by ligating Yellow Tang genomic DNA fragments enriched for a microsatellite motif into the *Hind* III cut site of pUC19 plasmid and electroporating the recombinant plasmids into *E. coli* strain DH5". After a recovery incubation in SOC broth, glycerol was added to a level of 20% of the final volume. The libraries should be stored at approximately -70°C. To isolate colonies for sequencing, cells from the glycerol stock should be spread on blue-gal/IPTG/ampicillin (BIA)-LB agar plates (or X-gal/IPTG/ampicillin plates).

Text sequences and electropherograms provided with these libraries

Edited text files (flanking plasmid sequences removed) that correspond to the enclosed electropherograms are on the PC-formatted diskette included in this envelope. The text file names correspond to the information on the electropherograms: a GTCA library is designated "B", a TACA library

is “C”, and a TAGA library is “D.” For future reference, the *Hind*III restriction sequence is AAGCTT. In pUC19 recombinants, the 3' *Hind*III restriction site is followed immediately by GCAT (we used the forward primer described below for sequencing).

Selecting clones for sequencing

Although these libraries have been prepared from genomic DNA fragments 350 to 700 bp long, post-screening procedures and digestions may have resulted in a few inserts that are less than 300 bp long. Smaller inserts are less likely to have sequences flanking a microsatellite that are long enough for primer design. Additionally, non-recombinants may not develop blue precipitate fully, and it is possible to select two colonies simultaneously if they are not well separated on the plate. For these reasons, determining the size of an insert prior to selecting clones for sequencing may be desirable.

We determined the size of the inserted fragments for these clones by PCR, using M13-cloning site primers (the sequences we use are provided below). We use sterilized toothpicks to transfer white colonies from the spread stock plates onto a blue-gal/IPTG/ampicillin LB (BIA-LB) plate which has a transparent grid taped to the bottom (samples enclosed). This plate is incubated overnight, and colonies are selected from this plate rather than from the original spread. Some colonies may subsequently develop faint blue pigmentation; we find that they generally contain recombinant plasmid with an insert of proper size.

Cells are scraped from a colony and heated to 100°C in a PCR tube that contains the PCR master mix. This lyses the cells and releases the plasmids. Polymerase is then added and the insert is amplified. PCR product is separated by electrophoresis on a 3% agarose gel and sized by comparison with Phi-X/*Hae* III standard. The insert size is about 110 bp less than the PCR product due to plasmid sequences that lie between the 5' ends of the primers and the cloning site at each end of the insert. The forward primer also works well for sequencing; its 3' end anneals 14 bases before the beginning of the insert.

For PCR, we prepare a master mix sufficient for 110% of the number of reactions that will be carried out.

Master Mix:

1 X

3.388 :L H₂O
1.0 :L 10X PCR Buffer
1.2 :L MgCl₂ (25 mM stock)
1.5 :L Sucrose/Cresol Red*
1.2 :L dNTP mix (stock w/all 4, 2.5 mM ea.)
0.75 :L Primer A [forward] (20 :M stock)†
0.75 :L Primer B [reverse] (20 :M stock)†
0.21 :L RNase A (20 mg/mL stock)
10.0 :L

* The Sucrose/Cresol Red solution is optional, but convenient. It acts as a loading buffer (giving density and color to the PCR mix) without inhibiting the reaction, as would glycerol or bromophenol blue, and allows loading of the gel directly from the reaction tube immediately after amplification. It consists of 10 mM cresol red in 20% sucrose 400.

† The DNA fragments are cloned into the *Hind* III (AAGCTT) cut site of the pUC19 plasmid, so any M-13 cloning site primers should work well. We find the following primers to be effective:

Primer A (forward): 5'- AGG AAA CAG CTA TGA CCA TG -3'

Primer B (reverse): 5'- ACG ACG TTG TAA AAC GAC GG -3'

Our typical annealing temperature for these primers is 57°C; they also work well at 58°C. We have not tested them above these temperatures.

Colony PCR from gridded colonies

Set a P-20 pipet for ca. 4 :L; attach a yellow tip, and scrape up the colony so that a small clump of cells is visible at the tip. (The cells may come directly from a colony, but, we prefer to first prepare a grid of cells as described above. If you choose to go directly from a non-gridded colony, see the description of "Hot Start" below for additional considerations.). Insert the tip into the reaction mix in the tube then slowly depress the pipet plunger and pipet up and down 4 - 6 times to suspend the cells in the reaction mix. Close the tube and place it on ice until all other tubes have been prepared. Overlay the reaction with 22 :L mineral oil. Place in thermal cycler and heat to 100°C for 10 minutes, followed by a 37°C indefinite soak. While the 100°C treatment is proceeding (this step lyses the bacteria), prepare a Hot Start solution:

Hot Start:	<u>1 X (Taq Polymerase)</u>	OR <u>1 X (Biolase)</u>
	4.425 :L H ₂ O	4.425 :L H ₂ O
	0.5 :L 10X PCR Buffer	0.5 :L 10X PCR Buffer
	<u>0.075 :L Taq (5 U/:L)</u>	<u>0.01875 :L Biolase</u>
	5.0 :L	5.0 :L

Of the two enzyme choices, we usually use BIOTAQ DNA Polymerase, available from Bionline USA Inc., Canton, MA, tel (781) 830-0360 (www.bionline.com). Note that the MgCl₂ supplied with BIOTAQ is 50 mM, and that the Master Mix protocol should be changed to accommodate any differences in stock concentrations.

NOTE: When we amplify directly from the non-gridded colonies, we use BIOTAQ DNA polymerase, double the amount of enzyme specified above, and increase the number of cycles listed below to 30. This should reduce the number of weak or failed reactions.

Once the cell lysing mixture has cooled to 37°C, open the PCR reaction tube and pipet 5 :L of the Hot Start mix beneath the mineral oil layer and directly into the reaction mix; pipet up and down 6 times to mix. Close all tubes and cycle as follows:

94°C 30"
57°C 30"
72°C 30"

Cycle a total of 21 times, then allow a 2 min. extension at 72°C, followed by a 5°C soak.

If Sucrose/Cresol Red is used, the reactions are now ready for loading onto a 3% agarose gel, 4 :L per reaction. If not, prepare a 2X loading buffer and mix 4 :L with 4 :L of the PCR reaction. We size against a Phi X/Hae III size standard. The red color from the cresol red tends to be rather faint; therefore, we usually add -2 :L of a typical 10X glycerol/bromophenol blue/xylene cyanol loading buffer to the end lanes of the gel to aid in gauging the progress of the electrophoresis. We run our samples on a standard mini-gel (-10 cm), and stop the electroporesis when the first dye front (bromophenol blue) is running off the end of the gel.

Standard PCR conditions for primer testing:

We use a standard set of conditions when testing primers. If we obtain satisfactory product, we may then adjust template concentration, annealing temperature, number of cycles, etc. Here is the recipe and conditions we use:

As noted, we have good luck with Biotaq, so we continue to use it. Sucrose/Cresol red can be omitted, but don't forget to add an additional 1:l water to compensate.

Reaction Mix:

Component	Stock Concentration	Final Concentration	Volume/Reaction
H ₂ O			5.15 :l
BioTaq Buffer	10X	1X	1.0 :l
Sucrose/Cresol Red (if testing on agarose)	10X	1X	1.0 :l
MgCl ₂	50 mM	2 mM	0.4 :l
dNTP's (premixed)	2.5 mM each	0.2 mM each	0.8 :l
Primers (premixed)	10 :M	varies, typically 0.6 mM (0.3 mM each)	0.6 :l
BioTaq	5 U/:l	0.025 U/:l	0.05 :l
Template DNA	2 ng/:l	0.2 ng/:l	1.0 :l
		Total	10.0 :l

Cycling Parameters:

Initial denaturation:

94EC 3 min

35 cycles:

94EC 40 sec

55EC 40 sec

72EC 30 sec

Final Extension:

72EC 4 min

Soak: <28EC

3. PCR of Yellow Tang Microsatellites.

Yellow Tang Microsatellite PCR conditions

Cocktail for 2 plates

Thermocycler program

Buffer	220 µl	1	95 ⁰ C x 3 min
MgCl ₂	220 µl	2	95 ⁰ C x 45 s
dNTPs	220 µl	3	55 ⁰ C x 45 s
P1	220 µl	4	72 ⁰ C x 45 s
P2	220 µl	step 2-4 x 29	
Taq	110 µl	5	72 ⁰ C x 3 min
Water	440 µl	6	4 ⁰ C

P1 = Forward modified microsatellite primer

P2 = Reverse microsatellite primer

- a. First round PCR - 10 µl reactions using 2.5 µl of template.
- b. Add 190 µl water (1/20 dilution) to PCR reactions (these can be frozen).
- c. Use 2 µl of this as template for nested PCR using labeled M13 Forward primer and unlabelled microsatellite-specific Reverse primer (P2). Cocktail is for standard 10 µl PCR as above.
- d. Electrophoresis dilution is about 1/50 of the labeled second round PCR reaction.
- e. Electrophoresis conditions are for standard microsatellite run.

4. Microsatellite electrophoresis on ABI3100 automated genetic analyzer.

5: Data analysis.

Each microsatellite will be tested for Hardy-Weinberg equilibrium. Phylogenetic relationships will be determined using both maximum parsimony and neighbour-joining methods. Maximum parsimony analysis will be performed using the branch and bound algorithm in PAUP (v.4.0b10 – Swofford, 2002). The trees will be rooted with an outgroup consisting of mitochondrial sequence from *Z. scopas*, a close relative of the Yellow tang. The data will be bootstrapped using 1000 random permutations and branches retained that occur at least 50% of the time. Trees will be constructed from the genetic distance with the neighbour-joining algorithm (Saitou and Nei, 1987), and again, the data bootstrapped (1000 replicates) using the MEGA computer program (Kumar *et al.*, 1993).

The Frequency of each allele in each population (geographic sample set) will be calculated. Analysis of molecular variance (AMOVA, Excoffier *et al.*, 1992) will be carried out using ARLEQUIN (version 1.1; Schneider *et al.*, 1997) to test for population subdivision. This will allow us to estimate the proportion of genetic variation that is distributed within and among populations.

B. This proposal is a partnership/joint venture between Washington State University Vancouver and the West Hawai'i Aquarium Fish Project (WHAP). Dr. Marc Noakes is the Principal Investigator, a postdoctoral research associate at WSU Vancouver. Co-investigators on the project include Dr. Brian Tissot and Dr. Ruth Phillips (WSU Vancouver), and Dr. William Walsh (Department of Aquatic Resources). The salmonid genetic laboratory at WSU Vancouver will provide full access to all equipment and reagents used in a wide range of molecular genetic approaches, including DNA extraction, agarose gel electrophoresis and documentation, PCR (including primer design and synthesis), DNA sequencing and all software analysis. The laboratory has access to an automated sequencer on the main WSU campus at Pullman, WA. Further, the WSU Vancouver Science department is actively pursuing the purchase of its own automated capillary sequencer to support this work as well as other projects. Dr. Phillips has considerable experience in salmonid phylogenetics and is familiar with the analysis of mt DNA sequence and RAPD banding patterns using the software described under objective 3, section 3.4. Working in conjunction with WHAP surveys, we will leverage considerable research effort. Dr. Tissot and Dr. Walsh are Principle Investigators of the WHAP and have extensive knowledge of the coral reef ecology of Hawai'i. Dr. Walsh is kindly providing the fin material from samples collected as a part of a WHAP study. These samples will also be made available for other, ongoing WHAP projects. Dr. Tissot is available to lend his expertise in local coral reef ecology, management policy and biological distribution analysis.

6. Findings

- A. The following data has been generated but not yet published;
1. DNA sequence of approximately 100 microsatellite clones from the library.
 2. Approximately 270 oligonucleotide PCR primer sequences have been developed for Yellow tang microsatellites. Of these, 34 have been synthesised for use in fluorescent PCR analysis.
 3. Eight Yellow tang individuals have been tested for each of the 34 synthesised primers and examined for polymorphism (see attached example agarose gel electrophoresis).
 4. Nine of the above PCR primers have been selected and analysed by fluorescent PCR analysis in 400 juvenile Yellow tang samples and 120 adult Yellow tang samples.
 5. Three PowerPoint presentations have been prepared, presented, and copies submitted to HCRI.
- B. The microsatellite typing results for the juvenile yellow tang analysis is being finalised.
- C. A subsequent proposal has been submitted to HCRI by Jeff Eble *et al.* The proposed research will utilize the microsatellite library and markers identified and developed by this project. It will extend the findings of this research by sampling a wider geographical area.

7. Evaluation

- A. Describe the extent to which the project goals and objectives were attained. This description should address the following:
 - 1. The goals and objectives outlined in the project proposal were attained with the exception of the statistical analysis. Due to extended delays during the relocation of Noakes from the USA to Australia, the statistical analysis of all microsatellite data generated during the project is currently being analysed. The statistical analysis is expected to be concluded within 3 months.
 - 2. There were no modifications made to the goals and objectives.
- B. Dissemination of Project results:
 - 1. All raw data has been submitted to HCRI and NOAA for archive.
 - 2. Public seminar presentations have been submitted to HCRI and have been included on the HCRI website for public dissemination and display. Results and microsatellite sequences are submitted to the Genbank international DNA database and will be published upon conclusion of the statistical analysis.
 - 3. The microsatellite sequences and genetic marker information has been prepared in manuscript form and will be published upon conclusion of the project. A second manuscript describing the identification of microsatellites in surgeon fish is in preparation and will be submitted at the conclusion of the outstanding analysis. A third manuscript is expected to be prepared using the findings of the statistical analysis.

Prepared By:  - 04/25/2005
Signature of Principal Investigator Date

COP Project Final Report

NOTICE

Subsequently, all NOAA COP recipients with approved grants will be asked to file a COP Project Final Report in the specified format upon expiration or termination of grant support. Consistency in reporting requirements for competitive research grant programs is desirable and this is behind COP's efforts in proposing a standardized format. The use of the Project Final Report format will provide the level of detail required to evaluate the effort invested by investigators and staff on project management; any actual accomplishments and research findings; and what goals and objectives were attained. The proposed final report format is compatible with the format in use by other agencies that participate in joint projects with COP, e.g. the National Science Foundation.

Public reporting burden for this collection of information is estimated to average 600 minutes per response, including time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed and completing and reviewing the collection of information.

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